The role of early life freshwater tolerance and combined stressors in limiting freshwater colonization in Atlantic Canadian stickleback species.

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Abstract

Atlantic Canadaian stickleback fishes (*Gasterosteidae*) differ in the salinities they inhabit; Threespine (*Gasterosteus aculeatus)* and fourspine (*Apeltes quadracus)* sticklebacks inhabit salinities from freshwater to saltwater, while blackspotted sticklebacks (*Gasterosteus wheatlandi*) and the "white" threespine stickleback ecotype (*Gasterosteus aculeatus*) are restricted to brackish or saltwater when breeding. I investigated if blackspotted and white threespine distribution might be limited by low freshwater tolerance during early life or when combined with cold temperatures, as occurs during over-wintering. I found blackspotted sticklebacks have relatively poor freshwater fertilization success, but all species had similar freshwater survivorship, development, and embryonic metabolic rates. Upon exposure to warm (18°C) and cold (4°C) freshwater and saltwater, adult blackspotted and threespine sticklebacks demonstrated similar standard and active metabolic rates, survival, growth, and hepatic protein synthesis rates. My results suggest blackspotted freshwater colonization might be limited by fertilization, but not by early life freshwater tolerance or the cold-stress combination as adults.

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Table of Contents

1. Introduction

1.1 When Might Physiology Limit Species Distributions?

A fundamental ecological characteristic of a species is the geographic range across which it is distributed, which may change over time (Gaston 1996; Gaston and Fuller 2009). Furthermore, successful range expansion into novel environments requires organisms to cope with a new set of abiotic and biotic variables, which can be energetically expensive if this new environment is more physiologically stressful than the original (Duckworth 2008; Slayter et al. 2013). The earliest theories surrounding range limitation hypothesized that species' ranges were largely determined by biotic factors at lower latitudes and altitudes and by abiotic environmental factors at higher latitudes and altitudes (Darwin, 1859). However, subsequent theoretical work incorporating interactions among species, temporal and spatial environmental heterogeneity and evolutionary dynamics suggested that a suite of interacting evolutionary and ecological processes can constrain an organisms' range (Sexton et al. 2009; Alexander et al. 2022). In particular, dispersal limitations, competitive exclusion by previously colonizing species, limitations in fitness due to abiotic tolerances and biotic interactions, and genetic swamping preventing local adaptation at range limits are among the factors predicted to limit a species' range (reviewed by Sexton et al. 2009; Lee-Yaw et al. 2016). Empirical studies similarly show that both abiotic and biotic variables, and interactions among these types of ecological variables, are associated with range limits, but the specific mechanisms underlying limits often remain unclear (Sexton et al. 2009; Lee-Yaw et al. 2016; Siren et al. 2019**)**. However, in some cases, abiotic variables have a greater impact on habitat suitability than biotic factors (King et al. 2020; Gonzalez-Salazar et al. 2013). For instance, the terrestrial slug *Deroceras panormitanum* invaded the Southern Ocean's

Marion Island and dispersed into a gapped and patchy distribution due to the species' tolerance of substrate salinity levels (Lee et al. 2009).

Moreover, abiotic environmental conditions are rarely static within an ecosystem and species often vary greatly in their tolerance to this variation (Bozinovic et al. 2011; Schulte 2014). While some species have incredibly broad tolerances to abiotic factors, others have very narrow tolerance ranges. The intertidal zone, for example, presents one of nature's most extreme routine fluctuations in environmental conditions, and to survive, inhabitants must be able to maintain cellular homeostasis at very high and low temperatures, oxygen levels, salinities and bouts of air exposure (Somero 2002). Furthermore, distinct intertidal zonation leads to subregions that differ in the degree of environmental fluctuations and, thus, the extent of the range of physiological challenges organisms face (Somero 2002). While species inhabiting the lower intertidal zones tend to be primarily limited by biotic factors such as predation and competition, the dominating limitations in upper intertidal zones tend to be abiotic factors (Beukema and Flach 1995). Indeed, the intertidal upper limits are often established by the organism's physiological capacity to tolerate highly variable abiotic conditions, with the highest reaching organisms normally displaying broader thermal tolerances, greater desiccation resistance, and more robust biochemical processes, including protein repair, than sub-tidal species (Somero, 2002). Zooxanthellae, dinoflagellates that live in symbiosis with coral, are an example of a subtidal species with very narrow tolerance ranges that abandon their niche when absolute temperatures, heat exposure durations, and irradiance exposure become intolerable, causing coral bleaching (Fitt et al. 2001). Overall, the ability to maintain physiological homeostasis amid environmental change is essential for organisms to survive in variable ecosystems (Bozinovic et al. 2011). Thus,

species with larger abiotic tolerance capacities are predicted to have a larger geographic range (reviewed by Sexton et al. 2009; Lee-Yaw et al. 2016).

Understanding an organism's upper and lower abiotic tolerance limits, including acute tolerances and the capacity for acclimation, in combination with their ability to leave the current environment for a more suitable one and their potential for future adaption, may be useful for understanding future changes in range distributions (Bozinovic et al. 2011; Hendry et al. 2009; Kultz 2015; Somero 2012). One of the most compelling examples of how knowledge of current abiotic tolerance limits may predict future changes in ranges is the northward distribution shift of many marine species, ranging from phytoplankton and macroalgae to marine mammals, as the climate changes (Hastings et al. 2020). As their historically optimal habitats experience rising temperatures and declining pH and oxygen levels, species have shifted their ranges into cooler habitats according to their abiotic tolerance limitations (Perry et al. 2005; Brennan et al. 2016; Hastings et al. 2020). A greater tolerance range may equip species to expand their distribution and take advantage of ecological opportunities presented by novel habitats (Lee and Bell 1999; Calosi et al. 2010; Bozinovic et al. 2011). Thus, taking a physiological trait-based approach, in combination with ecological understanding, may help biologists to better understand the abiotic factors that influence an organism's habitat choice and overall species range limitations in the present and future (Calosi et al. 2010; reviewed by Bozinovic et al. 2011).

An example of such expansion into new habitats, made possible by shifting climatic variables, is the colonization of freshwater by ancestrally marine species (Lee and Bell 1999; Hendry et al. 2009; Calosi et al. 2010; Bozinovic et al. 2011). Novel access to freshwater environments was created across much of the Northern hemisphere during the Pleistocene, when retreating glaciers altered the landscape. Post-glacial lakes were inhabited by ancestrally marine

animals, then faced isostatic rebound and heavy rainfall, resulting in freshwater environments (reviewed by Lee and Bell 1999). A range of species have colonized freshwater, including a plethora of invertebrates, such as hydroids (*Cordylophora sp.*), zebra mussels (*Dreissena polymorpha*) and crustaceans, and some fishes (Lee and Bell 1999). In North America many marine and diadromous fish species colonized freshwater lakes following the retreat of Pleistocene glaciers ~10-12,000 years ago (Lee and Bell 1999). Because freshwater systems differ from marine habitats in a range of abiotic and biotic factors, only those species with some existing physiological tolerance of this novel habitat could expand into these post-glacial freshwater systems (reviewed by Lee and Bell, 1999). The colonization of freshwater by marine fishes is a useful case in which to study if, and how, tolerance of abiotic factors such as salinity, temperature, and water oxygen content might facilitate colonization in some species while limiting range expansion in others.

1.2 What Limits Freshwater Colonization in Fishes?

The colonization of freshwater by marine organisms requires the ability to cope with a host of environmental challenges, including more dramatic fluctuations in temperature, changes in diet, and life in a low salinity environment, all of which are predicted to potentially limit range expansion (Lee and Bell 1999; Holker et al. 2004). Relative to the ocean, temperate freshwater systems consist of small volumes that are particularly susceptible to atmospheric influence, which produces habitats with low thermal inertia (Morash et al. 2021). As a result, temperate freshwater habitats experience more dramatic thermal variation in the form of both inter-seasonal extremes and within-season fluctuations (Morash et al. 2021). Further, unlike marine habitats, winder-induced icing over of freshwater lakes and ponds can cause hypoxia within the water body (Morash et al. 2021). Overall, cold winters are particularly challenging for ectothermic

fishes, in which body temperatures match environmental temperatures, reducing metabolic rate, appetite and energy availability, altering behaviour, and changing cellular membrane composition and fluidity, thus challenging osmo and iono regulatory capacity (Volkoff and Ronnestad 2020; Buhariwalla et al. 2012).

Salinity, or the concentration of dissolved salts in water, is the predominant abiotic difference between freshwater (0 ppt) and marine (~30-35 ppt) environments and broadly influences cellular and biochemical processes (Kultz 2015). Teleost fish must maintain an internal solute concentration of ~260-360 $mOsmkg^{-1}$, which is drastically lower than the salinity of seawater (35 ppt/ 1000 $mOsmolkg^{-1}$) and higher than the salinity of freshwater (0-0.2 ppt/ 0-15 $mOsmolkg^{-1}$) (Edwards and Marshall 2013; Hill et al. 2018; McCormick et al. 2013). In saltwater, fish face a diffusive ion gain and osmotic water loss, while the opposite is true in freshwater, where fish face diffusive ion loss and osmotic water gain (Edwards and Marshall 2013; Hill et al. 2018; Zimmer and Perry 2022). If not actively regulated, the diffusive and osmotic pressure of both saltwater and freshwater are lethal to living organisms, causing critical cellular dehydration or swelling, respectively (Edwards and Marshall 2013; Hill et al. 2018; Zimmer and Perry 2022).

Counteracting the ionic gradients between the internal and external environment in seawater and freshwater requires different, and in fact opposite, strategies, both of which can be energetically costly to maintain. Osmo and iono regulation in seawater or freshwater requires environment-specific physiological specializations in key ionoregulatory tissues, such as the gut, kidneys and gill. In particular, the function of ion-transporting cells, termed ionocytes, varies between salinities. In the gill, ionocytes facilitate active expulsion of ions and water conservation in marine fish and active uptake of ions and excess water expulsion in freshwater (Edwards and Marshall 2013; Hill et al. 2018; McCormick et al. 2013). Most fish species can only osmo and

iono regulate within a narrow salinity range as they are not able to effectively remodel osmoregulatory tissues to function in both salinity extremes (i.e., freshwater or seawater) (Edwards and Marshall 2013; Zimmer and Perry 2021; Davenport and Sayer 1993; Peterson and Meador 2008). Indeed, the habitat restriction that confines fish species to either saltwater or freshwater is largely attributed to the limited salinity range within which a species can osmo and ionoregulate (reviewed by Dymowzka et al. 2012; Zimmer and Perry 2021).

Freshwater and marine habitats also vary in trophic communities and, thus, a fish's potential diet (Ishikawa et al. 2019; Kainz et al. 2009), and the energetic resources required for growth and reproduction (Volkoff and London 2018; Hemre et al. 2002; Gatlin 2010). In particular, invertebrate prey contain fewer essential omega-3 fatty acids, such as docosahexaenoic acid (DHA), in freshwater than in marine habitats, which may limit fish growth and survival (Ishikawa et al. 2019; Kainz et al. 2009; Ishikawa et al. 2021). Indeed, DHA deficiency may prevent some teleost fishes from successfully colonizing freshwater (Ishikawa et al. 2019; Ishikawa et al. 2021). As well, maternal nutrition has been linked with the strength of teleost egg chorions, the nutritional quality of embryos' yolk sacs, and, consequently, the viability of her offspring (Perez-Atehortua et al. 2023). Overall, obtaining proper nutrition is critical to success in a new habitat (Ishikawa et al. 2021; Lee and Bell 1999).

To maintain homeostasis in freshwater, additional energetic resources may be spent maintaining iono- and osmoregulatory balance (Ern et al. 2013). In addition, at poleward latitudes, this occurs while in a colder environment with reduced nutritional resources (Smith and Thorpe 1976; Lamson et al. 2009). Fish undergo indeterminate growth and, as ectotherms, their growth rate is highly dependent on environmental conditions. Because energy is first allocated to fuel basic biological functions that are necessary for survival, such as cardiovascular function

and ion regulation, fishes generally experience low growth rates and elevated mortality rates over winter compared to summer due to cold-induced slowing of metabolism and limited food availability (Hurst 2007; Handeland et al. 2008; Reid et al. 2022; Gibbons et al. 2017; Middleton et al. 2024). The amount of energy required to fuel basic biological requirements, termed the standard metabolic rate, is a fraction of the maximal metabolic rate, which describes the maximum amount of energy that an organism is capable of synthesizing and using. The difference between maximal and standard metabolic rates is termed the "aerobic scope"; expendable energy that can be allocated to functions beyond basic maintenance costs, such as growth, reproduction, and escaping predators (Norin and Clark 2015; Rees et al. 2024). When an organism experiences sustained environmental stress, more energy must be dedicated to basic maintenance costs (Somero 2002). Consequently, as metabolism ramps up to meet the elevated energetic demands, the standard metabolic rate may increase, or may decrease if the organism fails to meet the new demand. Either way, sustained environmental stress decreases the aerobic scope, potentially reducing growth rate (Somero 2002).

For the majority of euryhaline fishes, growth capacity is highest in marine or brackish conditions, and is limited in freshwater. Some studies suggest that one reason for this is an increased metabolic cost of iono and osmoregulation in freshwater, leaving less energy leftover to allocate to growth; however, this appears to be species and environment specific (reviewed by Ern et al. 2014). While salinity is a major divider of aquatic habitats, it is not the only factor that differentiates freshwater and marine habitats in nature. Marine and freshwater habitats differ in the biological communities they foster and several abiotic conditions. Thus, euryhaline fish encounter many variable elements between saltwater and freshwater, and their performance in each habitat may be attributed to factors beyond salinity. Indeed, growth is likely limited in

freshwater habitats by nutritional constraints and further limited, in northern latitudes, by energetic tradeoffs mandated during harsh winters. In temperate regions, marine habitats provide more nutritionally complete diets than freshwater systems (Gross et al. 1988). Thus, the cost of survival and the capacity of the energetic budget determine the energy available for growth and reproduction. A fish's ability to grow and mature directly impacts their fitness, as improved growth increases fecundity and population persistence (Arendt 1997; Burgess and Bueno 2021; Pincheira-Donoso and Hunt 2015; Lampert and Trubetskova 1996; Mortita and Takashima 2005).

Under most circumstances, fish preferentially use protein for growth, making protein synthesis a relevant metric for growth (Walton and Cowey 1982; Kim et al 2012). Protein synthesis, and growth in general, is energetically expensive (Boeuf and Payan 2001), so synthesis rates generally correlate positively with metabolic rate and often decrease when energy production is limited, as occurs during hypoxia or fasting, although responses do vary between species (Boeuf and Payan 2001; Middleton et al. 2024; Lewis et al. 2007). For instance, in response to food limitation, the Brook Char (*Salvelinus fontinalis*) decreases protein synthesis rates in multiple tissues, and this effect is exacerbated in cold temperatures (Middleton et al. 2024). In response to hypoxia, the Arctic Char (*Salvelinus alpinus*) does not alter protein synthesis rates, while the Crucian Carp (*Carassius carassius*) significantly decreases protein synthesis rates (Ducros et al. 2023; Smith et al. 1996).

Overall, the colonization of freshwater requires an organism to overcome a host of challenges throughout their life history, including, and perhaps especially, during their most vulnerable life stages, where they must grow and reproduce despite variable nutrition, harsh temperature, and hypoosmotic conditions. From here on in, my thesis will focus on the challenges associated with

freshwater, with emphasis on how the freshwater challenge is exacerbated during vulnerable life stages.

1.3 Teleost Fish Ionoregulation Across Life History Stages and Seasonal Changes

1.3.1 Ionoregulation

Adult fish possess several specialized ionoregulatory tissues to help them counter the salinity gradient, including the kidney, urinary bladder, digestive tract, and gills (Edwards and Marshall 2013; Kultz 2015). In saltwater, where environmental salinities are higher than that of the fish's extracellular fluids, fish must take up water by drinking seawater, absorbing water through the digestive tract, and filtering urine at the kidney to produce a small amount of isotonic urine with little water content to minimize water loss (Edwards and Marshall 2013; Hill et al. 2018; Kultz 2015). In saltwater, fishes also ionoregulate by actively powering the efflux of Na^+ and Cl^- from the gills. In freshwater, fish must reduce water absorption during digestion, excrete large volumes of dilute urine, and ionoregulate by taking up Na^+ and Cl^- at the gills (Edwards and Marshall; Hill et al. 2018; Kultz 2015; Beyenbach 2004).

Although multiple tissues are integrated to play a role in osmoregulation, the gill ionocytes are the primary site of monovalent cation and anion transport (Edwards and Marshall 2013; Zimmer and Perry 2021; Evans et al. 2005; Dymowska et al. 2012; Zimmer et al 2014; Zimmer et al. 2017). In both high and low salinities, countering the dramatic ion gradient is an energetically demanding process, which requires ionocytes to be packed with mitochondria to satisfy the ATP demand of ionoregulation (Dymowska et al. 2012). The gills are also the primary site of gas exchange, acid-base balance, and nitrogenous waste excretion in fishes, potentially

leading to trade-offs in physiological functions (Edwards and Marshall 2013; Zimmer and Perry 2021).

Colonization of freshwater habitats requires that an organism be able to spend their entire lifetime in freshwater, including during spawning and early life stages, when fish tend to be quite vulnerable to environmental stressors. In addition, the relatively low thermal inertia afforded by freshwater systems results in extreme thermal variation that is not experienced within the ocean, including dramatic seasonal differences that expose freshwater inhabitants to harsh winters (Morash et al. 2021).

1.3.2 Effects of environmental salinity on fertilization success

Successful colonization of freshwater habitats requires successful reproduction, which, in turn, requires gametes to be viable in freshwater. Both sperm and eggs from euryhaline fish are often functional within a narrower salinity range than that in which adults a can ionoregulate, thereby potentially limiting the environment in which a fish can reproduce (e.g., McCormick et al. 2013). Indeed, the ability to successfully reproduce in freshwater seems to limit range expansion into freshwater habitats in several fishes (Lee and Bell 1999; Bell and Andrews 1997). For example, euryhaline anadromous fishes, who reproduce and spend their early life in freshwater, have colonized freshwater (e.g., landlocked Rainbow Trout and Atlantic Salmon) more commonly than catadromous species, who leave freshwater to reproduce and develop in the ocean (Bell and Andrews 1997). Freshwater fertilization is also challenging for many non-anadromous euryhaline fishes; gulf killifish (*Fundulus grandis*) acclimated to 7 ppt have ~40% fertilization success in freshwater, but ~80% at their acclimation salinity (Brown et al. 2012).

These fertilization declines in freshwater may be related to osmoregulatory failure of eggs and/or sperm in low salinity environments. In externally fertilizing fishes, such as sticklebacks,

sperm experiences a drastic osmotic shock when it is released into the water (Browne et al. 2015; Takei et al. 2012; Herrera et al. 2021; Cosson et al. 2008; Alavi and Cosson 2006). Sperm released by a freshwater fish is activated by hypo-osmotic shock, while saltwater fish sperm is normally activated by hyperosmotic shock (Browne et al. 2015; Takei et al. 2012; Herrera et al. 2021; Cosson et al. 2008; Alavi and Cosson 2006). However, while an osmotic shock is needed to activate sperm, too great a shock can have negative effects that ultimately impair gamete function. Indeed, the round goby (*Neogobius melanostomus*), native to freshwater and brackish regions of the Baltic Sea, has been a successful invader of North American freshwater systems but remains absent from marine habitats, potentially because sperm function is reduced in marine salinities (Green et al. 2019). The osmotic state is also important to the viability of eggs. For instance, eggs of the freshwater-spawning delta smelt (*Hypomesus transpacificus*) have a reduced activation capacity above 0.4 ppt, resulting in decreased fertilization success (Romney et al. 2019). The challenges associated with fertilizing offspring in freshwater may prevent species from successfully reproducing and, thereby, prevent freshwater colonization.

1.3.3. Osmo and ionoregulation during embryogenesis

Once fertilized, embryonic fish must survive in the environment for days prior to the development of functional osmoregulatory organs (Section 1.3.1; Fridman 2020; Varsamos et al. 2005). For example, gills do not begin to establish ionoregulatory function until several days post-fertilization (dpf), ranging from as soon as 3dpf in tilapia to as long as 25dpf in the ayu (Rombough 2007). Prior to the existence of ionoregulatory tissues, embryos rely on cutaneous ionocytes that are rich in mitochondria and ion transporters (Varsamos et al. 2005; Zimmer et al. 2017; Esaki et al. 2006; Parker et al. 2020; Fridman 2020). Ionocytes located on embryos' integument and yolk sac membrane (Figure 1.1) appear biochemically and functionally similar to

adult gill ionocytes (Fridman 2020). Although embryos are capable of regulating ion flux into and out of their bodies, cutaneous ion transport is less effective than gill-mediated ion transport, necessitating a functional shift to the gills as metabolism ramps up over development (Zimmer et al. 2014).

Surviving in salinities beyond a species' preferred range can require spending additional energy to maintain iono or osmoregulatory homeostasis, such that it can inhibit adult growth rate (Boeuf and Payan 2001). While adults may be able to manage such high energetic costs, embryos and juveniles, who lack specialized ionoregulatory tissues and are solely dependent on the yolk sac for nutrition, may not be equipped to maintain homeostasis in salinity extremes (Varsamos et al. 2005; Zimmer et al. 2014). Indeed, many anadromous fish species, which naturally spawn in freshwater, are quite sensitive to changes in salinity during fertilization and early embryonic life; when exposed to saltwater during embryonic development, many euryhaline anadromous fishes experience deleterious effects (DiMaggio et al. 2016; Winger and Lasier 1994; Morgan et al. 1992; Brown et al. 2012; Wagner 1967; Tay and Garside 1974). For example, despite migrating into marine environments during adulthood, embryonic exposure to saltwater causes death among striped bass (*Morone saxatilis*), pink salmon (*Oncorhynchus gorbuscha*), chum salmon (*Oncorhynchus keta*), coho salmon (*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*), and sockeye salmon (*Oncorhynchus nerka*) (Winger and Lasier, 1994; Wagner 1967). Saltwater exposure also causes a 90% decline in survivorship of embryonic alewife (*Alosa pseudoharengus*) and blueback herring (*Alosa aestivalis*) compared to freshwater (DiMaggio et al. 2016) and causes the successful hatch rate of steelhead salmon (*Oncorhynchus mykiss*) to drop from 98% to 84% (Morgan et al. 1992). These studies into the early life freshwater tolerance of anadromous fish species demonstrate how fishes that are

euryhaline at specific life history stages can be vulnerable to salinity stress as embryos (DiMaggio et al. 2016; Winger and Lasier 1994; Morgan et al. 1992; Brown et al. 2012; Wagner 1967; Tay and Garside 1974).

Early life tolerance to salinities outside the natural breeding salinity appears to be limited in some estuarine-spawning euryhaline fish species as well (e.g., Brown et al. 2012; Tay and Garside 1974). The gulf killifish (*Fundulus grandis*), a marsh-dwelling species that can survive and grow in freshwater during juvenile and adult stages (Burger et al. 2017; Ramee et al. 2015), demonstrates a 2-fold decrease in successful hatch rate when embryos are exposed to 0.4 ppt compared to 7 ppt (Brown et al. 2012). Embryonic common killifish (*Fundulus heteroclitus*), a species famous for their post hatch tolerance of salinities spanning 0.4 - 120 ppt (Joseph and Saksena, 1996), experiences a 77% drop in their successful hatch rate when embryos from marine-acclimated parents are exposed to freshwater compared to saltwater (Tay and Garside 1974). Further, embryos that did survive to hatch in freshwater experienced a reduced incubation duration, paired with a smaller size at hatch, suggesting freshwater interferes with proper development (Tay and Garside 1974). Together, these studies suggest that euryhaline fishes can

be more vulnerable to salinity stress during early life stages than during adulthood.

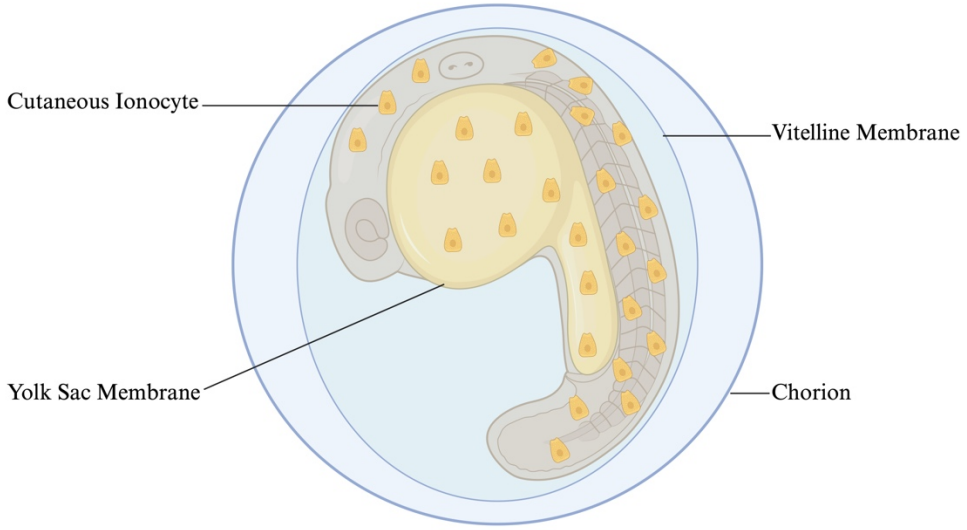

Figure 1.1. Illustration of cutaneous ionocytes on pre-hatch embryonic fish. Cutaneous ionocytes are responsible for ionoregulation in larval fish before the development of functional gills and additional osmoregulatory tissues (Fridman 2020). Created with BioRender.com

1.3.4 Ionoregulation and Temperature Change

The high degree of influence that atmospheric temperature has on water temperature in freshwater systems results in significant thermal fluctuations, causing the temperature of freshwater habitats to experience more dramatic seasonal differences than the ocean (Morash et al. 2021). Coping with these challenging temperatures can have negative impacts on an individual, particularly when the duration of such a fluctuation is long lasting (Morash et al. 2021). Fish inhabiting freshwater systems at temperate northern latitudes experience long lasting extreme cold during winter, often leading to high winter mortality rates (Morash et al. 2021; Hurst 2007; Shultz and Conover 1997).

Cold temperatures further exacerbate the challenge to maintain ionoregulatory homeostasis for two reasons. Firstly, chemical reaction rates and diffusion has a positive correlation with temperature; this physiological thermal dependence is generally quantified using the Q_{10}

coefficient, which describes the rate of change of an organism's resting metabolic rate as a result of a 10°C change in temperature (Chaui-Berlinck et al. 2001; Halsey et al. 2015). Thus, in the cold, the rate at which ions can be transported, against the concentration gradient, across the gills is reduced, especially for ions transported via temperature-sensitive enzymatic reactions (Buhariwalla et al. 2012). Meanwhile, diffusion is less temperature sensitive, such that passive ion loss remains constant when freshwater gets cold while the ability to counteract loss is inhibited (Buhariwalla et al. 2012). Secondly, cold temperatures alter the state of matter of the lipid bilayer enveloping fish gills, transforming the membrane's liquid state into a gel-like state (Buhariwalla et al. 2012; Snyder and Hennessey 2004). Fish cope with this phenomenon by employing a strategy known as homeoviscous adaptation, wherein they compensate for temperature-mediated change of membrane fluidity by changing the composition of their gill's lipid bilayer; in the cold, homeviscous adaptation primarily involves increasing the amount of polyunsaturated fatty acids and decreasing the amount of saturated fatty acids (Buhariwalla et al. 2012; Snyder and Hennessey 2004; Hazel 1984). Indeed, high levels of polyunsaturated fatty acids, and low levels of saturated fatty acids, in the lipid bilayer is a characteristic of Antarctic fishes (Storelli and Acierno 1998). Northern temperate fishes are found to have more polyunsaturated fatty acids in their gill's lipid bilayer, and less saturated fatty acids, during the winter relative to summer (Farkas and Csengeri 1976). For example, among freshwater alewife (*Alosa pseudoharengus*) populations that experience high winter mortality rates, individuals who die over-winter have been shown to have significantly lower levels of polyunsaturated fatty acids and higher levels of saturated fatty acids in their gill lipid bilayer relative to survivors (Snyder and Hennessey 2004). Snyder and Hennessey (2004) note that the winter mortalities among alewives were particularly deficient in docosahexaenoic acid (DHA) and speculate that the

ability to successfully use homeoviscous adaptation to compensate for the cold may rely on endogenous fatty acid synthesis via fatty acid desaturase enzymes.

However, the challenge of a temperate northern winter reaches beyond the effects of cold temperatures and interactions with environmental salinity. In addition to cold temperatures, these winters are characterized by food scarcity, although starvation is unlikely to be a dominant underlying cause of winter mortality (Johnson and Evans 1996; Jutfelt et al. 2020; Hurst 2007**)**. Indeed, Johnson and Evans (1996) found that overwinter mortalities of white perch (*Morone americana*) at 2.5°C were not caused by starvation and Snyder and Hennessey (2004) found that overwinter survival of alewife (*Alosa pseudoharengus*) was unaffected by the nutritional quality of their diet. In fact, many successful cold-adapted species behaviorally choose dormancy as an energy saving strategy in the cold, which includes refraining from food consumption (Speers-Roesch et al. 2018; Jutfelt et al. 2020; Reeve et al. 2022; Rowsey et al. 2024). When dormancy is combined with the cold-temperature induced slowing of the metabolic rate (Q_{10} effects), most fishes are predicted to have a decreased energy demand in winter (Johnson and Evans 1996; Reeve et al., 2022). Thus, the challenge of temperate northern winters may be more dependent on the inhibitory effect of cold temperatures on ionoregulation (Buhariwalla et al. 2012; Snyder and Hennessey 2004; Storelli and Acierno 1998; Farkas and Csengeri 1976).

1.4 Model system to test if abiotic tolerances limit freshwater colonization: The family *Gasterosteidae*

To understand if the colonization of freshwater by euryhaline fish is limited by salinity tolerance during fertilization, at early life history stages, and/or in combination with the added stress of cold winter temperatures experienced by adults, I used the sticklebacks (family *Gasterosteidae*) as a model group. Fish in the family *Gasterosteidae* include several euryhaline

species which have adaptively radiated from ancestrally marine or diadromous populations to colonize post-glacial freshwater lakes and rivers (see Figure 1; Lee and Bell 1999). In particular, marine or anadromous ancestral populations of the common threespine stickleback (*Gasterostus aculeatus*) have repeatedly, and independently, colonized freshwater (Schulter 1993; Lee and Bell 1999; Gibbons et al. 2016). As well, fourspine, ninespine, and brook stickleback are also commonly found in freshwater habitats (Nelson 1968). However, some Atlantic European stickleback species (i.e., fifteenspine stickleback), Atlantic Canadian stickleback species (i.e., blackspotted stickleback), and Japanese stickleback species (i.e., Japan Sea stickleback) have not colonized freshwater, remaining largely restricted to breeding in marine habitats (Figure 4, Campeau et al. 1984; Blouw and Hagen 1990; Gilhen 1974; Scott and Crossman 1967; Scott and Scott 1988). Thus, the family Gasterosteidae includes closely related species, some of which have colonized freshwater and others which have not, providing a unique comparative system in which to test whether a limited physiological capacity for freshwater tolerance may constrain freshwater colonization and, if so, the mechanisms that underlie these tolerance limits.

The ability to maintain homeostasis in freshwater as adults likely limit some stickleback species from colonizing freshwater habitats. When the saltwater-restricted Atlantic European fifteenspine stickleback is exposed to a salinity spectrum spanning seawater to freshwater, they reach 100% mortality within 2-3 days at ~4 ppt, and within 4 hours at 0 ppt (Raffy 1953). This low salinity-induced mortality was accompanied by mass increases associated with water influx, indicating a loss of ionic and osmotic homeostasis (Raffy 1953). Further, comparisons of the saltwater-exclusive Japan Sea stickleback and its close relative, the freshwater-colonizing Pacific Ocean stickleback (Figure 1.2) (Ishikawa et al. 2016; Ishikawa et al. 2019) have shown that adult Japan Sea Sticklebacks have a reduced ability to maintain homeostasis in freshwater after acute

exposure and lower survival in freshwater than the freshwater colonizing-Pacific Ocean stickleback (Ishikawa et al. 2016).

Recent studies comparing Japanese stickleback species also suggest that freshwater colonization may also be limited by availability of an essential nutrient, docosahexaenoic acid (DHA) (Ishikawa et al. 2019). Marine habitats provide sticklebacks with DHA-sufficient diets while freshwater habitats do not (Kainz et al. 2009; Ishikawa et al. 2019). Many freshwater stickleback populations have evolved extra copies of the *Fads2* gene, which encodes a key fatty acid desaturase and facilitates biosynthesis of DHA. Ishikawa et al. (2019) showed that when *Fads2* copies are added to the saltwater-exclusive Japan Sea stickleback via transgenics, their DHA content and survivorship in freshwater increases.

Lastly, freshwater colonization may be limited by an inability to successfully reproduce in freshwater. For instance, the fifteenspine stickleback is incapable of reproducing in freshwater because its sperm is rendered immotile upon contact with freshwater (Elofsson et al. 2003). Overall, these studies demonstrate poor freshwater tolerance capacities in marine-restricted stickleback species at various life stages via different mechanisms.

In this thesis, I aim to study mechanisms potentially limiting colonization in Atlantic Canadian Blackspotted Stickleback, for which adult freshwater tolerance does not appear to be the limiting factor. Unlike the Japan Sea and fifteenspine sticklebacks, adult Atlantic Canadian blackspotted stickleback can survive and mount the necessary 'freshwater acclimation' gill transcriptional response when acutely exposed to freshwater (tested at 18-20°C) (Bernhardsson 2021). Juveniles of this species can also survive in freshwater, but both threespine and blackspotted sticklebacks responded negatively to freshwater; freshwater induced high mortality rates among threespine sticklebacks, but not blackspotted sticklebacks, while blackspotted

sticklebacks experienced stunted growth and lethargy (Campeau et al. 1984). These data suggest that all stickleback species may be especially sensitive to freshwater as juveniles, but this does not appear to explain the lack of colonization in the blackspotted stickleback.

Additionally, an Atlantic Canadian ecotype of the common threespine stickleback, termed the 'white' threespine stickleback, has only been detected in marine habitats, but adult individuals can also tolerate acute freshwater exposure (tested at 18-20°C) (Bernhardsson 2021). However, little work has been done to understand how their ability to tolerate freshwater varies throughout their life history (Bernhardsson 2021); it is presently unknown how freshwater exposure affects the blackspotted and white threespine stickleback during reproduction, early life, and in adults at cold temperatures over winter.

Marine and freshwater ecotypes of Canadian Pacific threespine stickleback are both capable of surviving cold freshwater acclimation, but the freshwater ecotype has higher growth rates in freshwater than does the marine ecotype, suggesting strong selective pressure for growth in the cold in freshwater (Gibbons et al. 2016; Gibbons et al. 2017). Together, these data suggest that the combined stress of cold temperatures and freshwater exposure may also limit colonization in sticklebacks. Adult blackspotted and white threespine stickleback adults can survive freshwater exposure under summer temperatures, but it is largely unknown whether they can successfully compensate for exacerbation of the ionoregulatory challenge in freshwater when the challenge is exacerbated by cold-induced hardening of the gill membrane and slowing of ion transport (Buhariwalla et al. 2012; Snyder and Hennessey 2004; Storelli and Acierno 1998; Farkas and Csengeri 1976). If, indeed, fatty acid desaturases are important for overwintering in freshwater and DHA deficiency is associated with freshwater winter mortality (Snyder and Hennessey 2004), then the blackspotted stickleback would seem to be at a disadvantage compared to the

threespine stickleback as they possess only a single cope of the DHA synthesis enzyme *Fads2* (Ishikawa et al. 2019).

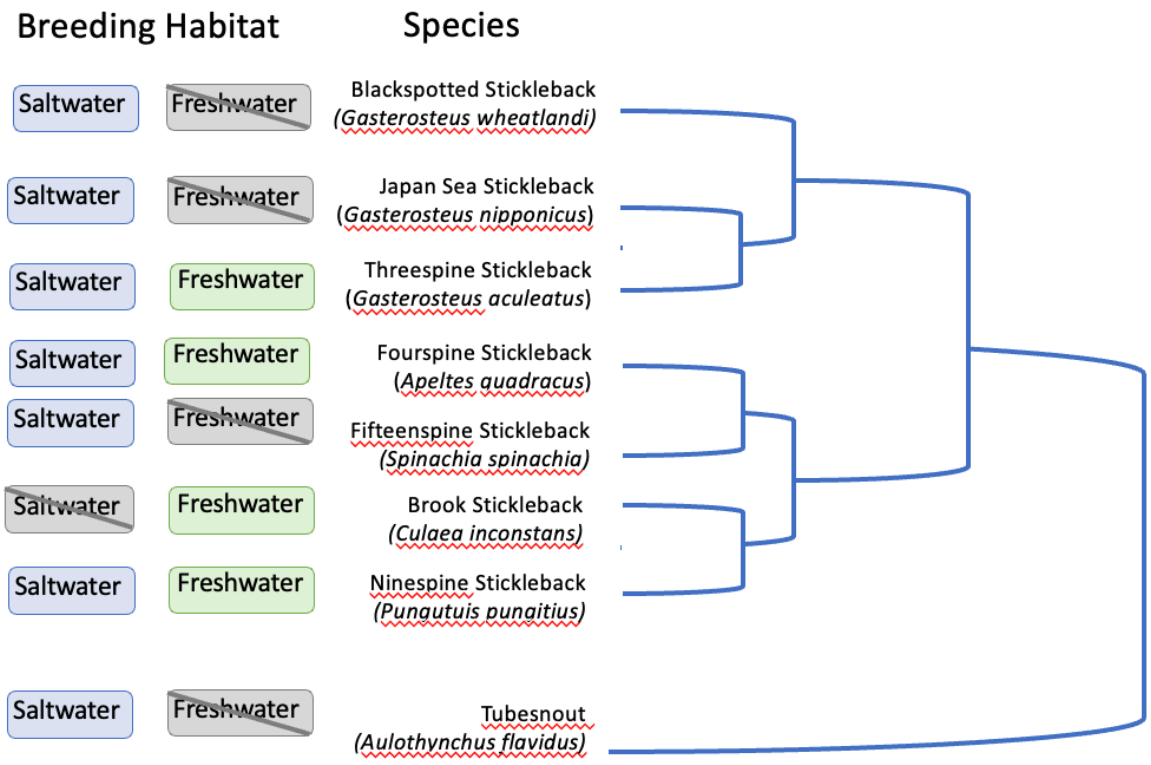

Figure 1.2. Stickleback phylogeny based on Lieu et al. (2023) with breeding habitat information from Gilhen (1974), Scott and Crossman (1967), Scott and Scott (1988).

1.5 Aims and Hypotheses of this Thesis

I took a comparative approach to better understand if, and how, freshwater tolerance across life history stages, and potential interactions with temperature, may limit freshwater colonization among Atlantic Canadian stickleback species. In particular, I studied species that vary in their freshwater colonization history: the freshwater colonizing common threespine stickleback (*Gasterosteus aculeatus*) and fourspine stickleback (*Apeltes quadracus*), the non-freshwater colonizing blackspotted stickleback (*Gasterosteus wheatlandi*) and the recently evolved (~10,

12,000 years ago; Samuk 2016) white threespine stickleback ecotype (*Gasterosteus aculeatus*) for which colonization ability is unknown. I hypothesized two non-mutually exclusive possibilities: 1) freshwater colonization is constrained by a limited capacity to reproduce in freshwater and to tolerate freshwater during early life stages, in particular, as embryos (e.g., Elofsson et al. 2003) and 2) freshwater colonization is constrained by the ability to survive and grow as adults amid the combined stressors of freshwater and winter-induced cold temperature (e.g., Gibbons et al. 2016, 2017).

I tested the first hypothesis of early life salinity tolerance limiting colonization using five metrics (Figure 2). I quantified fertilization rates in brackish water (10 ppt) and freshwater (0 ppt) and compared fertilization success rates between the common threespine stickleback, fourspine stickleback, white threespine stickleback, and blackspotted stickleback. I then reared fertilized embryos at 0 ppt and 10 ppt and quantified development time, incubation duration, and embryonic survival rates. At 3dpf, oxygen consumption rates were measured as a proxy for resting whole-body metabolic rate to reflect each species' baseline energy expenditure in saltwater compared to freshwater. I predicted that the freshwater colonizing common threespine and fourspine stickleback embryos would display similar fertilization rates, development times, survival rates, and oxygen consumption rates at both salinities while 0 ppt-reared embryos of the non-colonizing species would display lower fertilization rates (Eloffson et al. 2003; Palmer and Able 1987), stunted development (Dahlke et al. 2020), lower survival rates (Able and Palmer 1988), and higher metabolic rates (Boeuf and Payan 2001; Zimmer et al. 2014) relative to the 10 ppt reared embryos.

To test if the combined exposure to low temperatures and freshwater may limit freshwater colonization (*sensu* Gibbons et al. 2018), I exposed adult (colonizing) common threespine and

(non-colonizing) blackspotted sticklebacks to either brackish water (10 ppt) or freshwater (0 ppt) under winter temperature (4°C) or summer temperature (18°C). After two months of exposure, I measured adult standard metabolic rates, to estimate the cost of living in each treatment, and active metabolic rates, to assess each treatment group's escape ability and athletic capacity. Growth rates and mortality rates were measured over the course of the experiment, to infer each treatment group's ability to cope with their conditions, hepatic protein synthesis rates were quantified to give insight into the potential mechanism underlying growth capacity. I predicted that, under winter conditions, both the common threespine stickleback and blackspotted stickleback would experience moderate reductions in metabolic rates, growth rates, and hepatic protein synthesis rate as these are common thermal effects of cold temperatures (Volkoff and Ronnestad, 2020; Morash et al. 2021; Gibbons et al. 2017), and reduced HSI and GSI. However, within each temperature group, I predicted that the blackspotted sticklebacks in 0 ppt would display metabolic rates either markedly higher than those in 10 ppt, reflecting an elevated cost of living due to the added costs of ion and osmoregulation, or depressed, reflecting an inability to maintain homeostasis. Either way, I predict a metabolic impairment to result in reduced growth rates, survival rates, HSI, and GSI in 0 ppt relative to 10ppt. Conversely, I expected there to be no differences between threespine sticklebacks at 0 ppt and 10 ppt. Furthermore, I expected the freshwater-effects on the blackspotted stickleback to be exacerbated under winter conditions relative to summer conditions, implying that the combined stress of low salinity and low temperature limits freshwater tolerance and, thus, successful colonization of freshwater by the blackspotted stickleback.

2. Methods:

2.1 Fish Collection

Fish collections took place throughout the summer (May – August) of 2023. Blackspotted stickleback (*Gasterosteus wheatlandi*), white threespine stickleback (*Gasterosteus aculeatus*), and fourspine stickleback (*Apeltes quadracus*) were collected from Lawrencetown Beach Park (44°38'44.0" N 63°20'28.8" W) and Rainbow Haven Provincial Park (44°65’47.8” N, 63°42’13”0 W) under the Department of Fisheries and Oceans Canada Maritime Region Scientific fishing licence #343930. Common threespine stickleback (*Gasterosteus aculeatus*) were collected from Lawrencetown Beach park as well as from Antigonish Landing (45°38'03.3" N 61°57'01.8" W), under the Department of Fisheries and Ocean Gulf (SG-RHQ 23-061) Region Scientific Collection permit. Fish were collected from waters near shore using dip nets and minnow traps set in shallow water and left for 1-6 hours. Upon collection, fish were examined to identify species and sex, then transferred into a bucket containing water from the collection site that was aerated using a portable bubbler and refreshed to prevent overheating.

Fishes were transported back to Saint Mary’s University (SMU) within aerated buckets, enclosed in coolers surrounded by ice packs. At SMU, fishes were maintained in the Aquarium Facilities, where they were kept in 20-gallon tanks at 18°C in 10 ppt water made with Instant Ocean salt (Instant Ocean®, Blacksburg, VA, USA) on a 16L:8D photoperiod to match the natural photoperiod during summer breeding season. However, there is considerable variation in photoperiod during the breeding season, so photoperiods within 14L:10D – 16L:8D would work. 10 ppt served as a control salinity because it is approximately isosmotic to extracellular fluids in the fish, thereby requiring little energetic expenditure for ionoregulation and osmoregulation, and has been previously used for ionoregulatory experiments in these species (Bernhardsson 2021).

Water quality tests and water changes were done daily while the biofilter was becoming established, and then switched to one to three times per week to maintain stable ammonia (NH_3), nitrite (NO_2^-), nitrate (NO_3^-), and pH levels. Fish were fed live *Artemia* nauplii (Brine Shrimp Direct, Utah, USA) and frozen blood worms and mysis shrimp (Hikari®, Kyorin Co., Ltd). Animal care practices were approved by the SMU Animal Care Committee.

2.2 Fish breeding and Early Life Freshwater Tolerance Experiments

Early life freshwater tolerance capacity was assessed by comparing four different metrics in 0 ppt and 10 ppt: 1) fertilization success; 2) development time of embryos; 3) survival rates through embryonic development; and 4) metabolic rate of embryos.

2.2.1 Fertilization Success

F1 crosses were made by *in vitro* fertilization following standard methods (e.g., Dalziel et al. 2012). In brief, the lab-bred F1 generation was created using wild-caught fish (section 2.1) to test the effects of salinity (0 ppt or 10 ppt) on fertilization, development, and hatching success. Embryos were fertilized in either 0.03 ppt or 10 ppt water. Sperm was obtained by euthanizing a male *via* a lethal dose of sodium bicarbonate ($NaHCO_3$) -buffered MS-222 and testes were extracted and separated. Eggs were obtained by gently pressing along a gravid female's abdomen to expel the eggs, which were divided and placed into two petri dishes. One testis was then added to each of the two petri dishes and chopped up to release sperm, but testis did not come into contact with eggs at this point. 10 mL of water of the appropriate salinity was then added to the dish and the contents were thoroughly mixed for ~15 seconds to allow for fertilization. Petri dishes were then tilted to a ~45° angle to collect eggs and sperm and left for one hour before assessing fertilization status. Fertilization was re-checked 24 hours later, when the embryos were

one day post fertilization (1 dpf). The number of fertilized eggs / the total number of eggs * 100 was logged as the fertilization success rate (%) of each clutch.

Following fertilization, embryos were reared in their fertilization salinity and clutches were kept in plastic hatching cups with a mesh bottom, which sat inside a 20-gallon tank aerated with bubblers and treated with methylene blue following standard methods for threespine sticklebacks (e.g., Dalziel et al. 2012). Eggs were incubated at 18-20°C at a salinity of either 10 ppt or 0 ppt and dead embryos were removed from the hatching cups every 24-48 hours. Embryos were reared in the hatching cups until they emerged from the chorion, at which point they were transferred to new tanks and juveniles were fed First Bites (Hikari Tropical) in the mornings and live *Artemia* nauplii in the afternoons. Upon reaching adulthood (~4 months), fish were transitioned to one feeding per day of live *Artemia* nauplii, frozen blood worms and mysis shrimp to satiation. Due to low freshwater fertilization rates of the blackspotted stickleback, all subsequent experiments were carried out using clutches fertilized entirely in 10 ppt and then transferred to either 0 ppt or 10 ppt one hour after fertilization. Thus, all subsequent early life tolerance metrics for blackspotted, white threespine, common threespine, and fourspine sticklebacks assessed two groups that were both fertilized at 10 ppt and then reared at 10 ppt or 0 ppt from one-hour post-fertilization onwards (Figure 2.1).

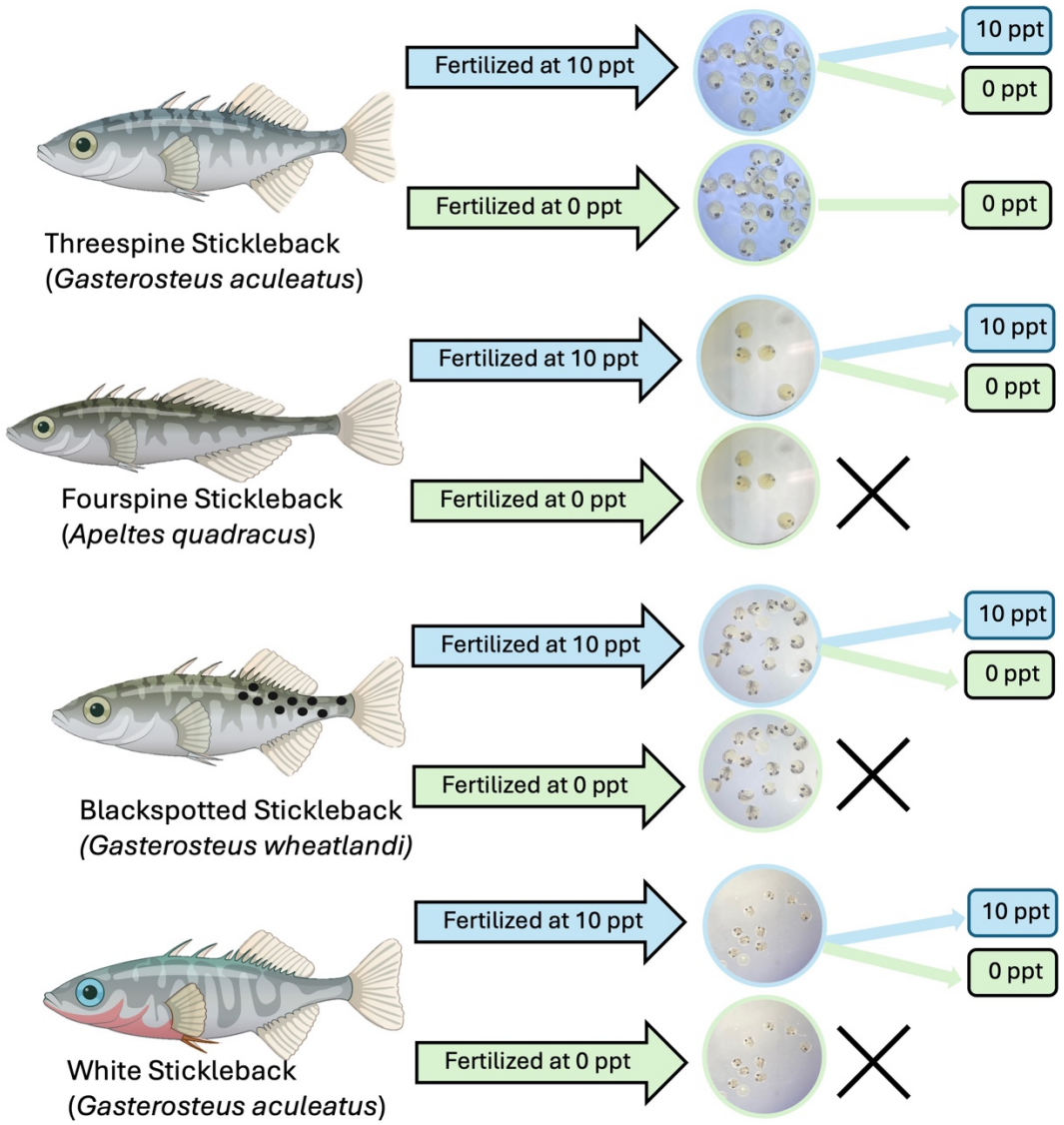

Figure 2.1. Experimental design used to test effects of salinity on common threespine, fourspine, blackspotted, and white threespine stickleback embryos after successful fertilization (Sections 2.2.2 to 2.2.4). Due to low freshwater fertilization success of the blackspotted stickleback, 0 ppt-fertilized offspring were not used in further experiments (denoted by "X"), with the exception of threespine sticklebacks. *In vitro* fertilization was completed at 10 ppt and after fertilization had taken place, clutches were split in half and incubated at 10 ppt or 0 ppt. Created with BioRender.com.

2.2.2 Development Stage of Embryos

Developmental stages were assessed based on Swarup (1958). Development time was recorded as the length of time that it took an embryo to reach the end of embryonic development (i.e., formation of mouth and tail and ready to hatch; stage 25 of Swarup, 1958) and were accurate to ± 1 day.

2.2.3 Survival to Four Days Post Fertilization

Embryonic survivorship was assessed at four days post fertilization (4 dpf) by counting the number of embryos that were alive at this stage. The number of surviving embryos at 4 dpf / the number of fertilized embryos * 100 was logged as the survival rate (%). Note that survival rate was assessed at 4 dpf also because at this point many clutches were split in half and preserved for additional experiments measuring embryonic ionocyte density (data not shown). The developmental stage (Swarup 1958) reached at 4 dpf was also recorded.

2.2.4 Embryonic Metabolic Rates

Oxygen consumption rates were measured as a proxy for whole body metabolic rate. When embryos were 3 dpf, their resting metabolic rate was measured using a custom 24-well microplate respirometry system (Loligo® Systems, Viborg, Denmark). Embryos were placed individually into one of the microplate's 80 uL glass wells (respirometer: organism size ratio ranged from 49.6 uL:1 um to 61.5 uL:1 um), leaving six wells empty to record background oxygen consumption. Each well was fitted with an oxygen sensor spot on the bottom of the well (Loligo® Systems, Viborg, Denmark) and filled with water of the appropriate salinity. Wells were sealed with PCR sealing film (BioRad, Mississauga, ON, Canada). Metabolic rate trials ran two microplates in parallel, with one microplate recording 0 ppt embryos and the other

microplate recording 10 ppt embryos. With each trial, microplates and oxygen sensors were alternated between 0 ppt and 10 ppt. Sealed Microplates were aligned atop their associated Microplate Reader, which measured oxygen content within the wells using an optical fluorescence oxygen system (Loligo® Systems, Viborg, Denmark). Microplates were covered with an opaque block to keep the plate in place and prevent disturbance. The microplate setup was placed on an orbital shaker (Thermo Scientific), on the lowest setting, to gently shake the plates and ensure oxygen was well mixed within each well. Metabolic rate trials were conducted within a climate-controlled room, which held water temperatures at 18°C. Metabolic rate was measured using MicroResp™ v1 automated microplate respirometry software (Loligo® Systems, Viborg, Denmark) to measure the oxygen decline within each well by sampling the oxygen saturation every second for two hours. Embryo oxygen consumption rates were calculated within the software as the slope of the oxygen decline.

Preceding each trial, microplate wells were rinsed with 95% ethanol (EtOH) to prevent bacterial growth and oxygen sensors were calibrated at 18°C and the salinity of the upcoming trial. To set the 0 % oxygen air saturation reading, each well was filled with a 0.32 M Na_2SO_3 solution, and values were recorded by MicroResp software (Loligo® Systems, Viborg, Denmark). To set the 100% oxygen air saturation reading, ~1 L of water of the appropriate salinity was aerated to match 100% air saturation with a bubbler for 30-45 minutes. The microplate wells were filled with this water and the oxygen values were recorded.

Following each trial, egg diameter was measured so that metabolic rates could be standardized by size (µm). Background oxygen consumption (average rate of the 6 blank wells) was subtracted the from metabolic rate of each embryo recorded on the associated microplate.

2.3 Simulation of overwintering conditions: Adult tolerance to the combined stress of freshwater and cold acclimation

2.3.1 Experimental Setup and Acclimation

Five families of juvenile 10 ppt-reared blackspotted stickleback, nine families of 10 ppt-reared common threespine stickleback, and five families of 0 ppt-reared common threespine stickleback were reared to adulthood following the early life salinity tolerance experiments (Section 2.2). 0 ppt-reared blackspotted sticklebacks were not used in further experiments because these groups (n =12 families) did not survive to the start of the combined stress experiment. Due to the fact that we did not record daily deaths, and the blackspotted family sizes were smaller than those of threespine stickleback, we could not determine if juvenile freshwater survival varied among groups. In November 2023, when fish were ~4 months old, they were tagged with unique colour identifier elastomer visible implantable elastomer (VIE) tags (Northwest Marine Technologies, Annacortes, WA, USA). All 10 ppt-reared fishes were transferred into experimental tanks, which, at the time of transfer, matched their rearing conditions (18°C; 10 ppt). 0 ppt fertilized and reared threespine stickleback families were transferred into treatment-specific holding tanks, which, at the time of transfer, matched their rearing conditions (18°C; 0 ppt).

I acclimated tagged juvenile threespine and blackspotted fishes to either: a) 18°C and 10 ppt, b) 18°C and 0 ppt, c) 4°C and 10 ppt, or d) 4°C and 0 ppt (Figure 2). Due a low sample size, blackspotted sticklebacks were only acclimated to the 4°C 0 ppt and 10 ppt treatments. 18°C experimental tanks were 20 L tanks kept on shelves within a temperature-controlled room that maintained their water temperature. 4°C experimental tanks were 20-gallon tanks kept within a water table, hooked up to a custom wet-table (Integrated Aqua Systems Limited, Vista,

California, USA) with two ½ hp recirculating chillers (Aqua Logic, Monroe, North Carolina, USA). The chiller system maintained the temperature of water surrounding the tanks at 4°C, thereby maintaining water temperature of the experimental tanks. Water temperature was checked daily with thermometers. Each treatment had three static experimental tanks containing 16-18 individuals. There were five to 30 individuals per family that were evenly distributed across treatments and, if possible, across experimental tank replicates. When multiple members of a single family existed within a treatment, the members' experimental results were averaged for a single data point per family per treatment.

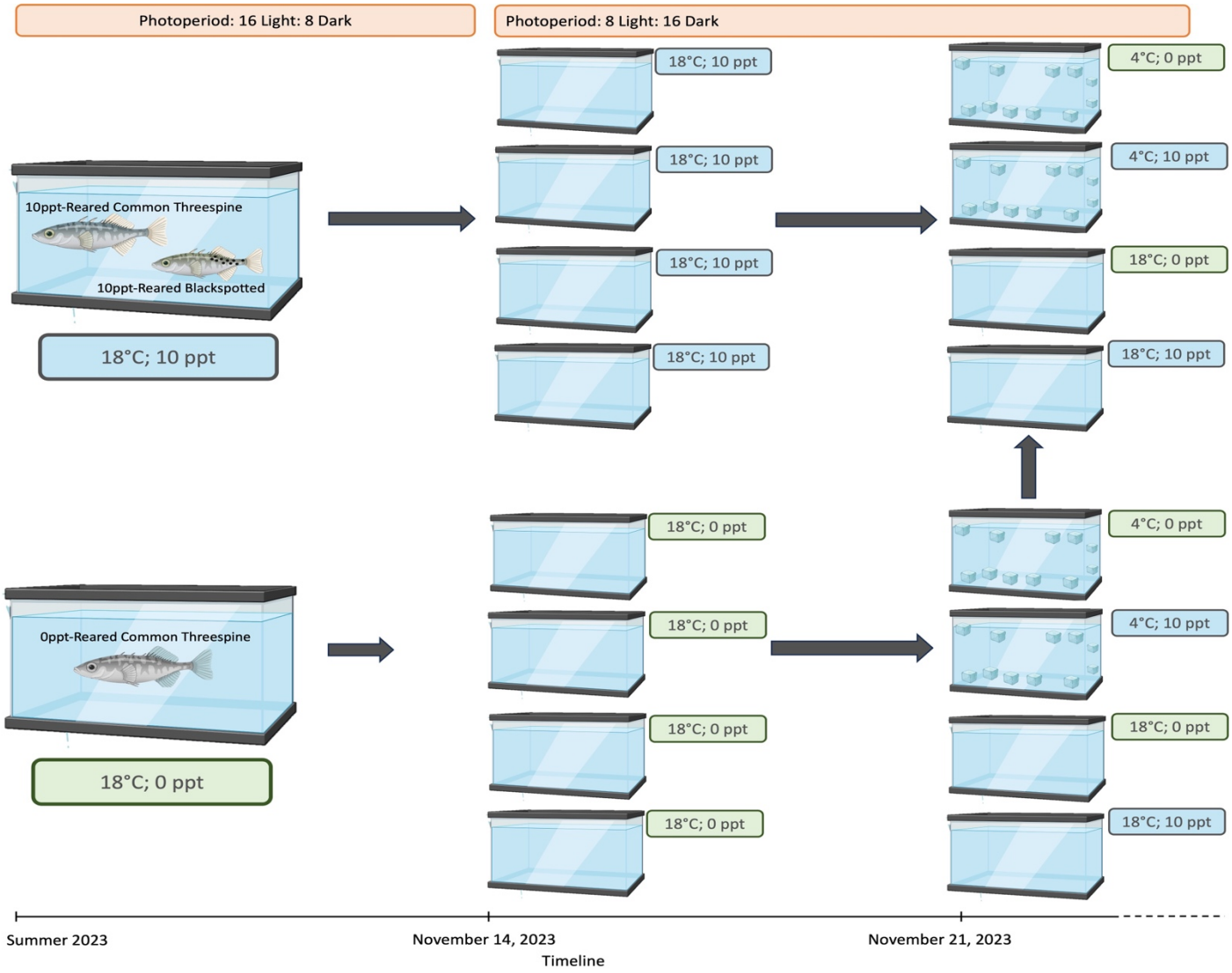

Figure 2.2. Experimental design used to test effects of salinity and temperature on adult threespine and blackspotted stickleback. Five families (n=5-7 individuals per family) of 10 ppt-reared adult Blackspotted and nine families (n=5-23 individuals per family) of 10 ppt-reared adult common Threespine sticklebacks were moved into treatment tanks that matched their rearing conditions. Five families (n=3-25 individuals per family) of 0 ppt-reared adult common Threespine stickleback were moved into treatment-specific holding tanks that matched their rearing conditions. Treatment tanks and holding tanks were gradually changed into the appropriate treatment conditions over the course of eight days by a rate of 1.25 ppt/day and 1.75°C/day. On the 8th day, when holding tanks and treatment tanks aligned on the final treatment conditions, 0 ppt-reared Common Threespines were transferred from holding tanks into the appropriate treatment tanks. Created with BioRender.com

Fishes were exposed to treatment conditions gradually, with the exception of photoperiod, which was changed from 16 L:8 D to 8 L:16 D on November 14, 2023 (see Figure 2). Abrupt change of photoperiod is common practice in similar experiments (Oldham et al. 2023; Taranger et al. 1988), although some studies opt for gradual change of photoperiod (Gibbons et al. 2016; Gibbons et al. 2017). I opted for the abrupt change for ease of experimentation and operating under the assumption that the acclimatory period was sufficient to allow the fish to acclimate to the photoperiod. However, while this assumption is commonplace, there is a lack of research as to whether fish respond differently when photoperiod is changed abruptly or gradually.

4°C experimental tanks and holding tanks were decreased from 18°C to 4°C over a period of 8 days (November 14 - 21, 2023), decreasing the temperature by 1.75°C/day. In the 0 ppt experimental tanks, salinity was decreased from 10 ppt to 0 ppt over a period of 8 days (November 14 - 21, 2023), decreasing the salinity by 1.25ppt/day (Palmer and Able 1987; Audet et al. 1986). In the 10 ppt holding tanks, salinity was increased from 0 ppt to 10 ppt over a period of 8 days (November 14-21, 2023), increasing salinity by 1.25 ppt/day. Once the holding tanks matched the conditions of their respective treatment, the 0 ppt-reared common threespine sticklebacks were removed from the holding tanks and distributed through the appropriate experimental tanks. Fishes were acclimated to their experimental conditions for two months prior to beginning experiments (late-November, 2023 – early-February, 2024).

2.3.2 Standard Metabolic Rate Measurements in adult fishes

Standard oxygen consumption rate was measured as a proxy for whole body standard metabolic rate to estimate basic energetic requirements under the various treatment conditions. Fish were fasted for 24 hours prior to standard metabolic rate experiments. Metabolic rate trials

were conducted using intermittent flow respirometry equipment (glass chamber Core Resting Respirometry System, Loligo® Systems). Oxygen consumption rates were measured in 16 mL custom glass chambers connected, by PVC tubing, to a recirculating pump, which was always on to ensure oxygen mixing within the chamber, and a flush pump, which switched on intermittently to refresh the chamber's water and replenish depleted oxygen. The respirometer, including tube and pump volume: fish body size ratio ranged from 8.4 mL:1 g to 114 mL:1 g. Oxygen consumption rates were checked for effects of allometric scaling, using a linear regression model (RCore Team 2021). Body mass did not have a significant correlation with mass-standardized metabolic rates across species ($\mu mol\ g^{-1}\ h^{-1}$; $p=0.09$; $R^2=0.03$), indicating that allometric metabolic scaling did not significantly impact my results (Supplementary Figure 1).

Chambers were outfitted with oxygen sensor spots (Loligo® Systems, Viborg, Denmark) on the upper surface in the middle of the chamber. For few fish, who weighed 0.15 grams or less, the flush pump created a current in the chamber that was too powerful for the fish to cope with. For these fish, a modified tubing setup, with a smaller tube diameter, was used to reduce flow. For all trials, a plastic mesh barrier was placed in the back of the chambers to prevent the fish from getting their tail caught in the outflow. The metabolic rate setup held chambers submerged in system water, which was set up in a flow through system such that water was recirculated between the metabolic rate system and the reservoir, which held 19 L of constantly chilling 4°C water or 7.5 L of room temperature-maintained 18°C water. The metabolic rate setup was surrounded by Styrofoam to help maintain the water temperature and to block external disturbance.

Fish were weighed prior to each trial so that oxygen consumption rate could be standardized by size (g). Fish were placed in the chambers during the afternoon and left

overnight to measure the standard metabolic rate. Metabolic rate trials were run using Autoresp™ v3.1.3 software (Loligo® Systems, Viborg, Denmark). Trials were run using intermittent flow, wherein there was a continuous loop of measurement periods and flush periods. Each trial ran four fish in parallel, but the respirometry setup prevented running multiple salinity and temperature treatments concurrently because the system could only draw water from a single reservoir. Trials rotated through treatments so that standard metabolic rate trials ran for every temperature and salinity treatment every week over the course of 12 weeks (February – April, 2024). When running experiments, fish were randomly selected but were generally size matched so that fish under ~1.5 grams were run together and fish above ~1.5 grams were run together. Preliminary trials showed that larger fish consume oxygen faster and reach hypoxic levels in the chamber (30% O_2) within the measurement period that is required to pick up a reliable oxygen consumption rate for the smaller fish. Therefore, larger fish were run on a shorter measurement period, such that oxygen levels never decreased below 50% O_2. Smaller fish were run on a measure-flush loop consisting of a 15-minute measure, where the chamber was sealed and oxygen decline was recorded; 10-minute flush, where the flush pump powered on and refreshed the chamber with aerated water (90-100% air saturation); and a 2-minute wait, where the chamber seals and allows time for oxygen readings to stabilize before recording the rate of decline. Larger fish were run in a 5-minute measure, 5-minute flush, 2-minute wait. Trials began in the afternoon and oxygen was measured every second by a Witrox 4 Oxygen Meter for Oxygen Sensors, using fluorescence signal detection (Loligo® Systems, Viborg, Denmark), with temperature, salinity, and barometric pressure compensation.

Trials were ended the following morning and fish were re-weighed before returning to their tank. A blank trial was also run following each trial, where each chamber was measured for

30 minutes to quantify background oxygen uptake. Glass chambers were rinsed with 95% ethanol (EtOH) after every other trial to reduce bacterial growth. Oxygen probes were calibrated weekly by filling chambers with 0.32 M Na_2SO_3 to set a 0% air saturation standard and by filling chambers with water aerated to 100% air saturation standard. The metabolic rate setup (including pumps, tubing, and ambient water reservoir) was rinsed thoroughly following every trial.

Oxygen consumption rates were calculated within the Autoresp™ v3.1.3 software as the lowest 10% of measurements within the 16-hour "night" window of their photoperiod. The slope of the oxygen decline was divided by the fish's body mass. Background oxygen consumption rates were accounted for by subtracting each chamber's blank oxygen consumption rate (always < 10% SMR) from the oxygen consumption rate of the fish run in that chamber immediately prior.

2.3.3 Active (Post-Chase) Metabolic Rate Experiment

Active metabolic rates were measured to estimate the upper metabolic limits, and infer the capacity for activity, of fish within each treatment. Active metabolic rates were measured over one week immediately following the conclusion of the standard metabolic rate experiment (April 15-22, 2024). Active metabolic rate trials were measured on the same individuals used for standard metabolic rate. Active metabolic rates were measured using the same intermittent flow glass chamber respirometry setup as standard metabolic rates (Section 2.3.1) and fish were re-weighed before and after trials. Active (or maximal) metabolic rates are generally quantified in one of two ways: using a swim tunnel, wherein the water flow is controlled to gauge maximum sustained swimming speed and oxygen consumption rates as water increases in a stepwise manner, or using a chase method, wherein fish are chased to apparent exhaustion then immediately placed in a respirometer to measure the post-exercise oxygen consumption rate

(Norin and Clark 2015; Rees et al. 2024). Theoretically, the two metrics might provide a similar result in some species of fishes (Rees et al. 2024) and swim tunnel respirometry is thought to be a better choice for species who naturally swim for prolonged periods while the chase method is a better fit for species who are not natural sustained swimmers (Norin and Clark 2015). I opted for the chase method due to time limitations, and because it provides insight into when the fish chooses to stop swimming; a correlate to ecological death (Rees et al. 2024).

Prior to measuring oxygen consumption, each fish was chased to apparent exhaustion in their treatment conditions. Fish were chased by hand and by a noise stimulus created by tapping on the side of the bucket. When the fish stopped responding to the chase stimuli, they were gently prodded or pinched on the tail. The chase procedure ranged from one to five minutes, continuing until the fish stopped responding to tail pinches and could no longer maintain an upright position against current in the water column. Once the fish reached apparent exhaustion, it was quickly transferred into the glass chamber (<30 seconds of air exposure) where oxygen consumption rate was measured for 5 minutes. At the end of the day's metabolic rate measurements, blank oxygen consumption rates were measured in each chamber for 30 minutes.

Oxygen consumption rates were calculated as the slope of the oxygen decline divided by individual body mass. Background oxygen consumption rates were recorded in a 30-minute measurement period in each chamber at the end of the day and was accounted for by subtracting each chamber's blank rate from fish oxygen consumption rates. Glass chambers were cleaned and calibrated as in section 2.3.2.

2.3.4 Mortality Rates of adult fish during temperature and salinity experiments

Over the course of the combined stressor experiment, the tank and species of every

mortality was recorded. The sum of the mortality data tracked the number of species' deaths per treatment.

2.3.4 Growth Rates

At the end of the combined stressors experiment, fish were weighed, and standard length was measured. Growth rates were calculated as growth per day by subtracting each fish's initial mass and length (prior to the combined stressors experiment) from their final mass and length (following the experiment). The difference was divided by the number of days spent in the experiment. Growth was calculated by day, rather than degree day, because all days over the course of the study were above the temperature cutoff for sticklebacks (3.0-3.5°C; Gibbons et al. 2017).

2.3.5 Hepatosomatic and Gonadosomatic Indices

At the end of the combined stressors experiment, the hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated for each fish to get a sense of energetic reserves (Chellappa et al. 1995) and how maturation and spawning capacity differed between treatments (Rizzo and Bazzoli 2020). Fish were euthanized using a lethal dose of sodium bicarbonate ($NaHCO_3$) -buffered MS-222 and the liver and gonads were extracted and weighed. The HSI and GSI were calculated as the mass of the liver (HSI) divided by body mass and the mass of the gonads (GSI) divided by body mass.

2.3.6 Hepatic Protein Synthesis Experiment

At the end of the combined stressors experiment, hepatic protein synthesis rates were measured, following the methods of Lamarre et al. 2015, to further assess potential mechanisms underlying any observed growth differences. Although the majority of protein synthesis-based

growth takes place in the white muscle (Krebs et al. 2023), preliminary experiments revealed very low protein synthesis rates in these species, such that somatic protein synthesis could be undetectable. Thus, I focused on liver to ensure detectability and because hepatic protein synthesis rates correlate with somatic protein synthesis rates and growth (Cassidy et al. 2016; Treberg et al. 2016). Fish were given an intra-peritoneal injection of a stable isotope labelled tracer: ring-D_5-phenylalanine. Injections used the flooding dose technique, where each fish was injected with a tracer volume that was 1% of their body mass (Lamarre et al. 2015). The tracer solution was made up of 50% 75 mM phenylalanine and 50% 75 mM D_5-phenylalanine according to Lamarre et al. (2015). Fish were weighed prior to injection, so that the tracer volume to be dispensed could be calculated as exactly 1% of their body mass. Fish were then placed into a water bath, of the appropriate treatment salinity, containing an anesthetic dose of $NaHCO_3$-buffered MS-222. Fish were anesthetized until unresponsive to a light tail pinch. To inject, a slit was cut in a sponge to hold the fish ventral side up while covering their gills and keeping the body moist. The sponge was soaked in the appropriate anesthetic solution (0 ppt or 10 ppt). Fish were placed upside down within the sponge and injected in their peritoneal cavity *via* the lower abdomen using a 10 μL, 32 gauge glass syringe (Hamilton Company). Once injected, the fish were placed into an aerated recovery tank which matched the temperature and salinity conditions of their treatment. Fish were left in their recovery tank for 240 minutes to allow for tracer incorporation.

Following tracer incorporation, fish were euthanized using a lethal dose of $NaHCO_3$-buffered MS-222. The liver was extracted, flash frozen in liquid nitrogen, and stored at -80° until analyzed for protein synthesis rates following methodology previously described by Lamarre et al. (2015). Briefly, liver samples were homogenized by sonicating in 1 mL of 0.2 M perchloric

acid (PCA) then centrifuging at 13,000 g for 5 minutes. The supernatant, containing unincorporated tracer, was recovered and set aside. The pellet, containing incorporated tracer, was re-homogenized in PCA and centrifuged 3 more times. Subsequent supernatants were discarded. The pellet was then re-suspended in 6 mL of 6 M hydrochloric acid (HCL). The pellet-HCL complex was incubated at 110°C for ~20 hours before adding 100 µL of 5 M potassium hydroxide (KOH) to precipite PCA from the unincorporated tracer samples (original supernatant). The mixture was then made acidic by adding 100 µL of 6 M HCL and centrifuging the mixture at 13,000 g for 30 seconds.

Following incubation of incorporated samples and HCL-acidification of unincorporated samples, the solution was passed through C-18 solid phase extraction chambers. 1 mL of 100% methanol (MeOH) was first passed through to clean the chamber, followed by 1 mL of 1 M HCL, 1mL of unincorporated samples or 2 mL of unincorporated samples, and 1 mL of HCL. All these effluents were discarded. The sample was eluted from the chamber using 250 µL of 30% MeOH, collected in an Eppendorf tube, and evaporated for 1-2 hours at 110°C. Solid phase extraction chambers were reused up to 5 times, but reuse was only within incorporated and within unincorporated samples, a single chamber was never used for both an incorporated and unincorporated sample.

Once all liquid was evaporated, the sample was resuspended in 75 µL of distilled water by vortexing thoroughly. 50 µL of the sample was then transferred to a GC-MS compatible vial, along with 20 µL of phosphate buffer and 130 µL of 26.1 g/L pentafluorobenzyl bromide in acetone solution. The mixture was vortexed and incubated at 60°C for 45 minutes. After incubation, 330 µL of hexane was added to the vials and the solution was vortexed thoroughly.

The solution then divided into organic and inorganic phases. 200 µL of the organic phase was transferred into a glass insert, which was placed into the vial, sealed, and read into the GC-MS.

2.4 Statistics

I took the average of all individual values within a family for each dependent variable, and used family averages in subsequent analyses. This was done because of the small number of individuals per family in some crosses, reducing power when running a nested-ANOVA with family as a random effect. I tested for an effect of species, salinity and/or temperature on response variables using RStudio (version 1.1.463) with R (version 3.6.1) and visualized using GraphPad Prism (v9.0.0). All data was initially tested for normality and homogeneity of variance. When data fit the necessary assumptions, I performed two or three-way analyses of variance (ANOVA) to assess the influence of the independent variables (salinity, species, temperature) and interactions among variables on response variables measured as indicators of freshwater tolerance. When data was not normally distributed, I fit data to more appropriate generalized linear models (gaussian, quasipoisson, binomial, or gamma models).

To assess the influence of salinity and species during early life, I analyzed fertilization and oxygen consumption rate data using a two-way ANOVA, analyzed development and incubation duration using a quasipoisson generalized linear model, and analyzed survival using a quasibinomial generalized linear model using the "lme4" package in R (v. 1.1-35.5).

During adulthood, I used generalized linear models, with gaussian distribution, to assess the influence of salinity, species, and temperature on metabolic rates, growth, and hepatic protein synthesis and used a binomial proportion generalized linear model (GLM) to assess the influence on mortality. When a significant effect of a given independent variable or a significant interaction effect was detected by the GLM, I conducted post hoc tests using estimated marginal

means pairwise comparisons to assess significant differences between groups using the "emmeans" package (v. 1.10.4) in R. Additionally, I used a binomial proportion generalized linear model to test for a tank effect on mortality rates.

3. Results:

3.1 Early Life Freshwater Tolerance

3.1.1 Fertilization Success

Fertilization success showed an overall effect of salinity ($F_{1,3} = 17.75$; $p < 0.001$), as well as a significant interaction between species and salinity ($F_2 = 4.12$; $p = 0.02$), such that blackspotted stickleback fertilization success was ~ 3-fold lower in 0 ppt than in 10 ppt ($p < 0.0001$). The blackspotted stickleback likely accounts for the overall salinity effect detected by the ANOVA as fertilization success was unaffected by salinity in the common threespine stickleback ($p=0.73$) and the white threespine stickleback ($p=0.99$). Fourspine Stickleback fertilization was not measured at 0 ppt due to small clutch sizes and there was no effect of species ($F_{1,3} = 2.09$; $p = 0.11$; Figure 1).

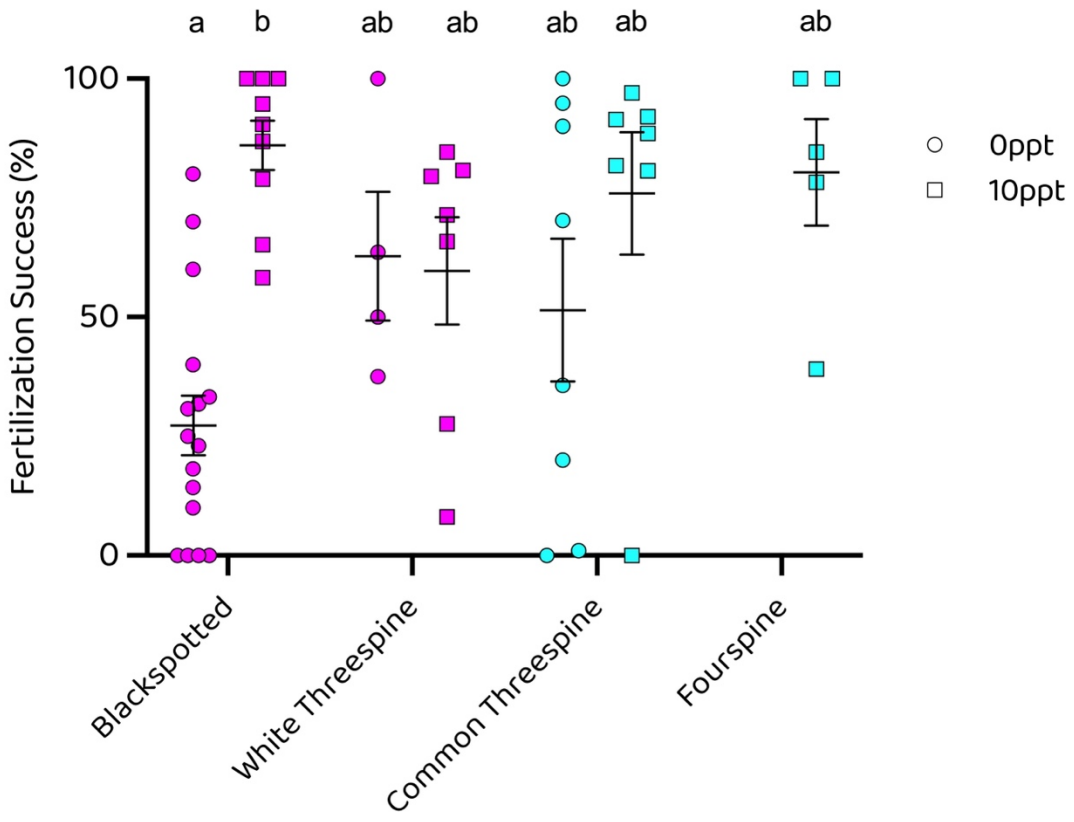

Figure 1. *in vitro* fertilization success of wild caught blackspotted stickleback (n = 9-16 families per salinity), white threespine stickleback (n = 4-6 families per salinity), common threespine stickleback (n = 7-8 families per salinity), and fourspine stickleback (n=5 families per salinity) clutches in freshwater (0 ppt; circles) and saltwater (10 ppt; squares). Fourspine fertilization was not measured at 0 ppt due to low sample sizes. Each data point represents the percentage of successful fertilizations for an individual family, with the central line representing the mean of all families ± SEM. Significant differences ($p < 0.05$) among groups, based on post hoc Tukey multiple comparisons tests, are represented by different letters at the top of the plot. Pink datapoints denote non-freshwater colonizing species while blue datapoints denote freshwater colonizing species.

3.1.2 Development Rate, Survival to Four Days Post Fertilization (4 dpf), and Development Time

The developmental stage reached by 4 dpf was not affected by salinity ($F_{1,3}$=0.01; p=0.28) or species ($F_{1,3}$=1.33; p=0.92), nor was there an interaction ($F_{1,3}$=0.64; p=0.59; Figure 2a). Survivorship to 4dpf was affected by salinity (p=0.001), but not by species (p>0.2), and there was no interaction (p>0.1; Figure 2b). Duration of embryonic development (i.e., from fertilization to hatch) was affected by species (F=14.0; p<0.001), but not by salinity (F=0.0; p=1.0), and there was no interaction (F=0.0; p=1.0; Figure 2c). Together, these data indicate there is not a species1.3-specific response to salinity during development.

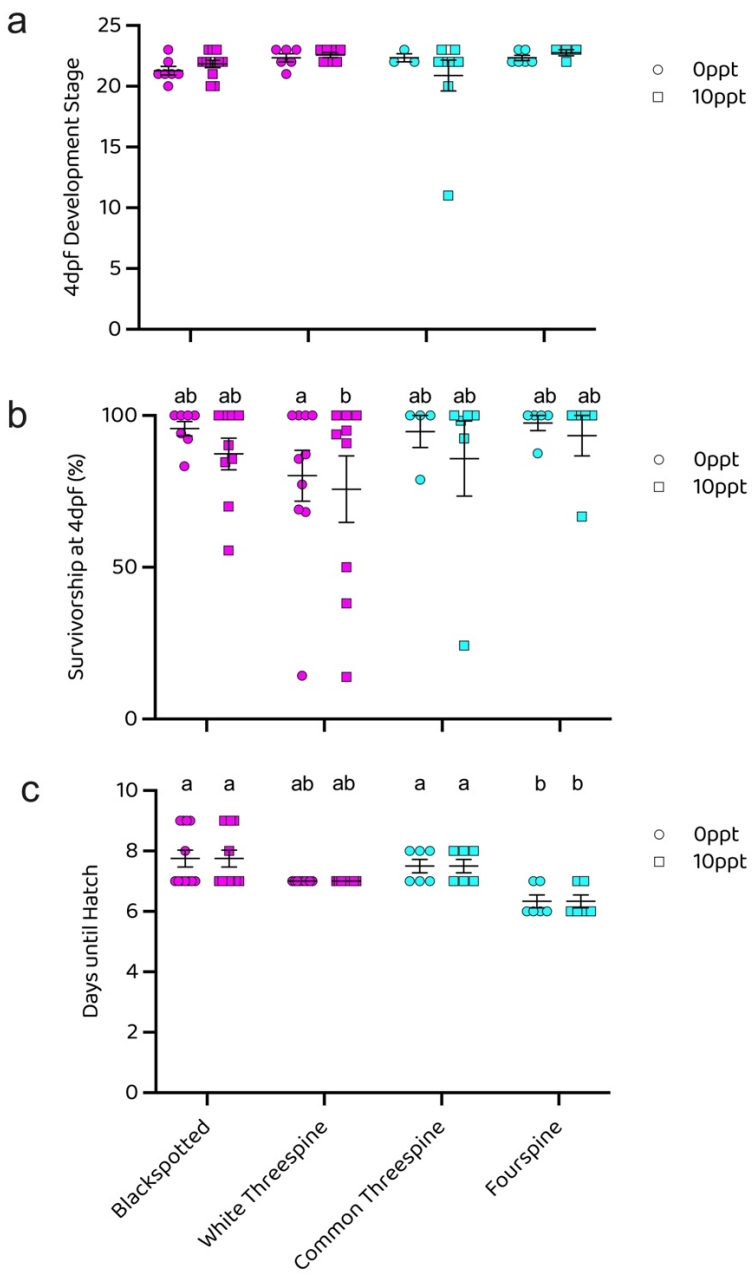

Figure 2. Average developmental stage at 4 dpf (panel a), survivorship rate to 4 dpf (b), and incubation time from fertilization to hatch (panel c) of blackspotted stickleback (n = 7-12 families per salinity), white threespine stickleback (n = 6-7 families per salinity), common threespine stickleback (n = 3-9 families per salinity), and fourspine stickleback (n = 4-6 families per salinity) clutches in freshwater (0 ppt; circles) and saltwater (10 ppt; squares). Each data point represents the average for an individual family, with the central line representing the mean of all families ± SEM. Significant differences ($p < 0.05$) among groups, based on post hoc estimated marginal means pairwise comparisons, are represented by different letters at the top of the plot. Pink datapoints denote non-freshwater colonizing species while blue datapoints denote freshwater colonizing species.

3.1.3 Embryo Metabolic rate

Embryo oxygen consumption rates differed significantly between species ($F_{1,3} = 4.85$, $p < 0.001$), but not between salinities ($F_{1,3} = 1.72$, $p = 0.20$). There was no interaction between species and salinity ($F_{1,3} = 1.50$, $p = 0.23$; Figure 3).

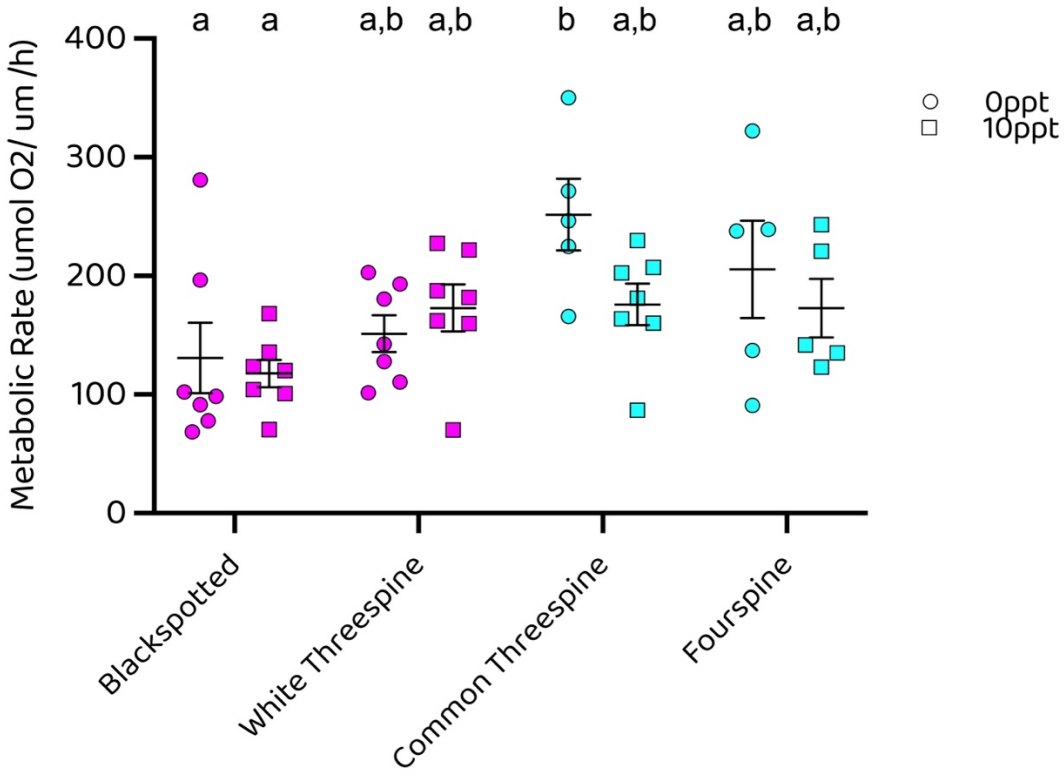

Figure 3. Metabolic rates, as estimated by oxygen consumption rates, of Blackspotted Stickleback (n = 7 families; 4-9 individuals per family), White Threespine Stickleback (n = 7 families; 2-10 individuals per family), Common Threespine Stickleback (n = 5-7; 4-14 individuals per family), and Fourspine Stickleback (n = 5 families; 4-9 individuals per family) embryos at 3 dpf in freshwater (0 ppt; circles) and saltwater (10 ppt; squares). Each data point represents the average oxygen consumption rate for an individual family, with the central line representing the mean of all families ±SEM. Significant differences ($p < 0.05$) among groups, based on post hoc Tukey multiple comparisons tests, are represented by different letters at the top of the plot. Pink datapoints denote non-freshwater colonizing species while blue datapoints denote freshwater colonizing species.

3.2 Adult freshwater tolerance: Combined Stress of freshwater and cold temperature

3.2.1 Standard Metabolic Rate of Adults Across Temperatures and Salinities

Oxygen consumption rates were significantly different between temperatures ($F_{1,2}$ = 55.92, p <0.001) but not between species ($F_{1,2}$ = 0.09, p = 0.91) or salinities ($F_{1,2}$ = 0.64, p = 0.46). There were no significant interactions between temperature, species, or salinity ($F_{1,2}$ < 2.45, p > 0.12; Figure 4a).

3.2.2 Active (Post-Chase) Metabolic Rate and Chase Duration of Adults Across Temperatures and Salinities

Active oxygen consumption rates did not differ between temperatures ($F_{1,2}$ = 4.03, p = 0.051), species ($F_{1,2}$=0.80, p = 0.46), or salinities ($F_{1,2}$ = 0.35, p = 0.56), nor was there an interaction between these factors ($F_{1,2}$ < 0.91, p > 0.32; Figure 4b). As well, chase duration did not differ between temperatures ($F_{1,2}$ =0.51, p = 0.48), species ($F_{1,2}$ = 0.62, p = 0.54), or salinity ($F_{1,2}$ = 0.59, p = 0.45), nor was there a significant interaction between factors ($F_{1,2}$ < 2.07, p > 0.16; Figure 4c).

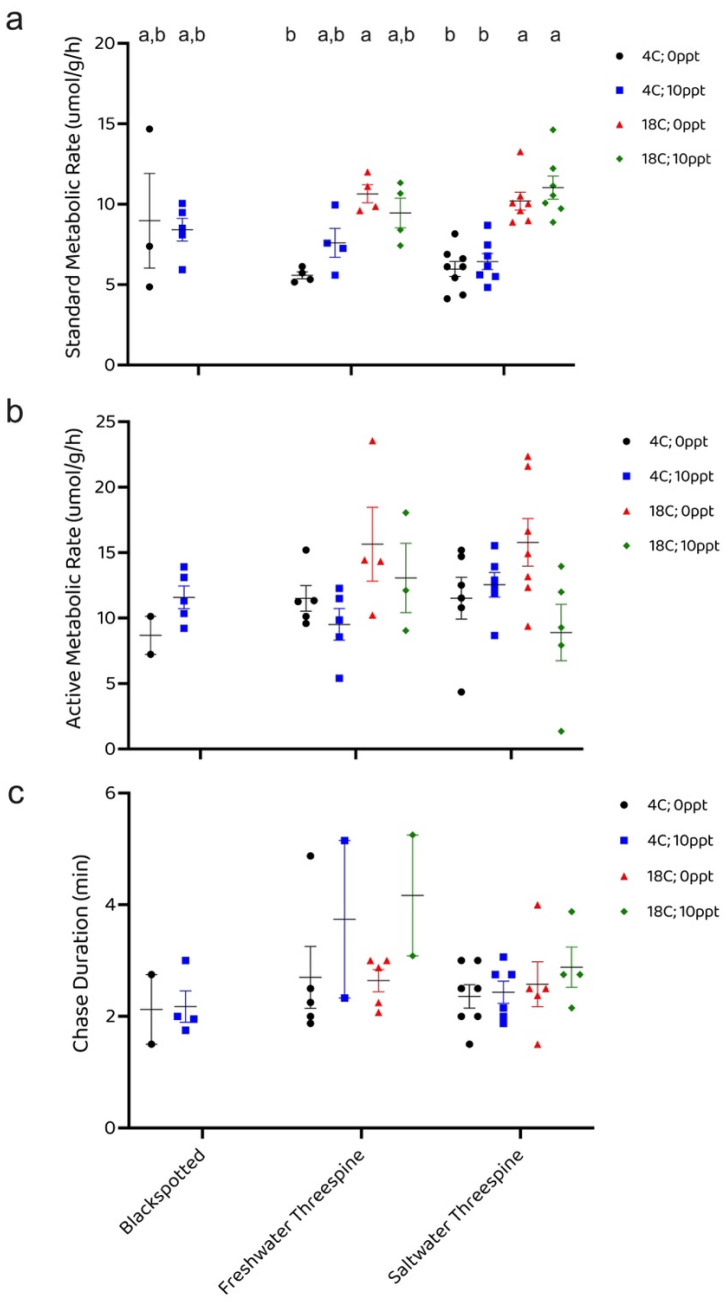

Figure 4. Standard metabolic rates (panel a) and active metabolic rates (panel b), as estimated by oxygen consumption rates, and chase duration prior to reaching apparent exhaustion (panel c) of blackspotted stickleback (n=5 families; 1-2 individuals per family), freshwater (0 ppt)-reared common threespine stickleback (n=4-5 families; 1-4 individuals per family), and saltwater (10 ppt)-reared common threespine stickleback (n=7-8 families; 1-4 individuals per family) adults in freshwater (0 ppt) and saltwater (10 ppt) at 4°C ('winter' treatment) and 18°C ('spring' treatment). Each data point represents the average for an individual family, with the central line representing the mean of all families ±SEM. Significant differences ($p < 0.05$) among groups, based on post hoc emmeans comparisons, are represented by different letters at the top of the plot.

3.2.3 Growth Rates

Growth rate by mass and length were significantly lower at 4°C than at 18°C (mass: $F_{1,2}$ = 57.33, p <0.0001; length: $F_{1,2}$ = 88.69, p < 0.0001). Growth rate by mass did not differ by species ($F_{1,2}$=1.36; p=0.27), but growth rate by length differed between species ($F_{1,2}$ = 8.09, p = 0.001), such that blackspotted stickleback had slower length-specific growth rates than the freshwater threespine (p=0.048) and saltwater threespine (p=0.004), but freshwater and saltwater threespines did not differ in either 4°C (p=0.96) or 18°C (p=0.50). There were no significant differences between salinities (mass: $F_{1,2}$ = 0.60, p = 0.44; length: $F_{1,2}$ = 0.13, p = 0.72), nor were there significant interactions between factors (mass: all $F_{1,2}$ < 2.21, all p > 0.14; length: all $F_{1,2}$ < 2.23, all p > 0.14; Figures 5a,b).

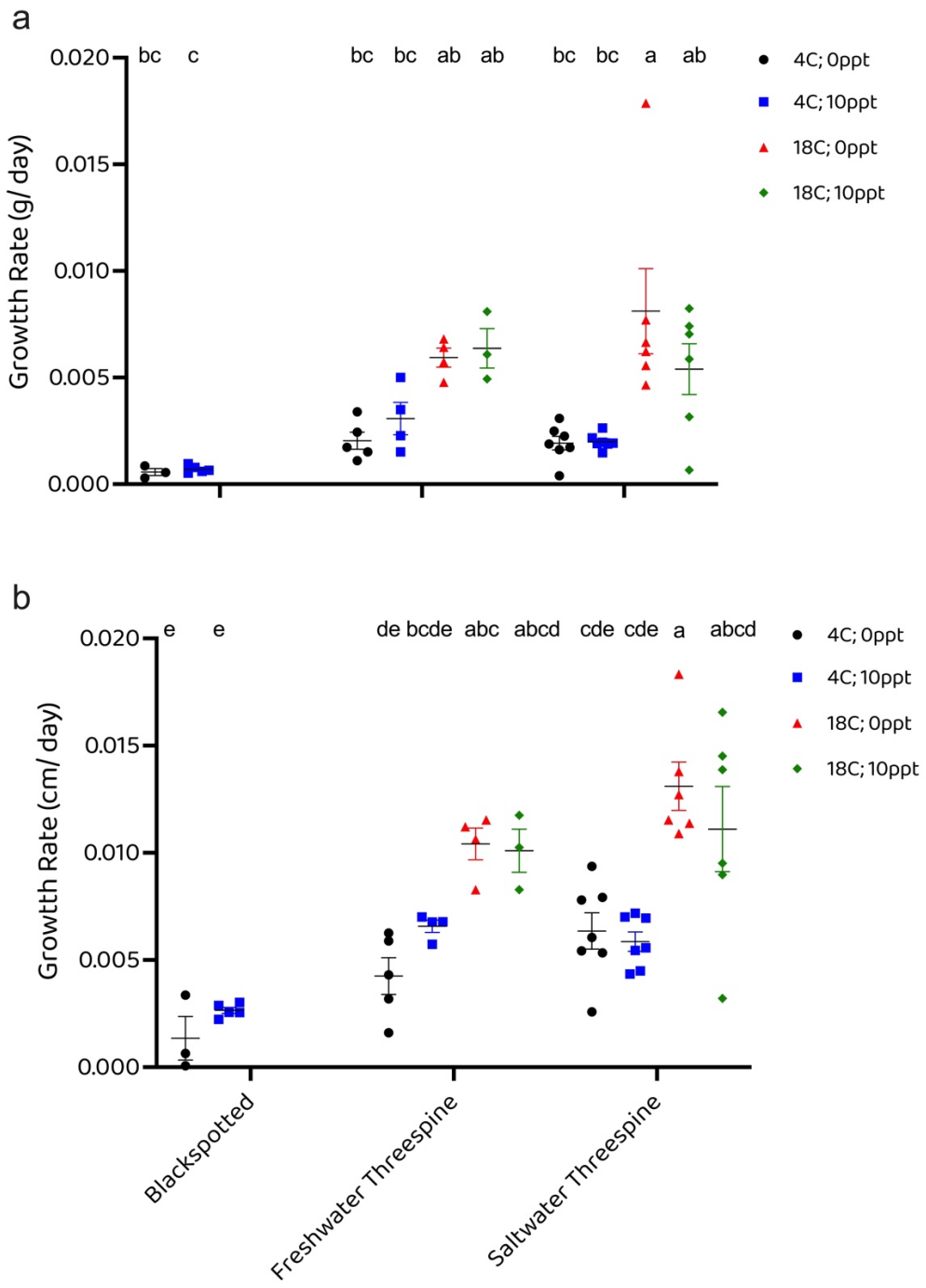

Figure 5. Growth rate by mass (panel a) and by length (panel b) of blackspotted stickleback (n=3-5 families; 1-3 individuals per family), freshwater (0 ppt)-reared common threespine stickleback (n=3-5 families; 1-4 individuals per family), and saltwater (10 ppt)-reared common threespine stickleback (n=6-7 families; 1-4 individuals per family) adults in freshwater (0 ppt) and saltwater (10 ppt) at 4°C ('winter' treatment) and 18°C ('spring' treatment). Each data point represents the average growth for an individual family, with the central line representing the mean of all families ±SEM. Significant differences ($p < 0.05$) among groups, based on post hoc Tukey multiple comparisons tests, are represented by different letters.

3.2.4 Hepatic Protein Synthesis Rate

Protein synthesis rates did not differ between temperatures ($F_{1,2} = 1.11$, $p = 0.30$), species ($F_{1,2} = 1.23$, $p = 0.31$), or salinity ($F_{1,2} = 0.14$, $p = 0.71$), nor was there a significant interaction between factors (all $F_{1,2} < 1.26$, all $p > 0.23$; Figure 6).

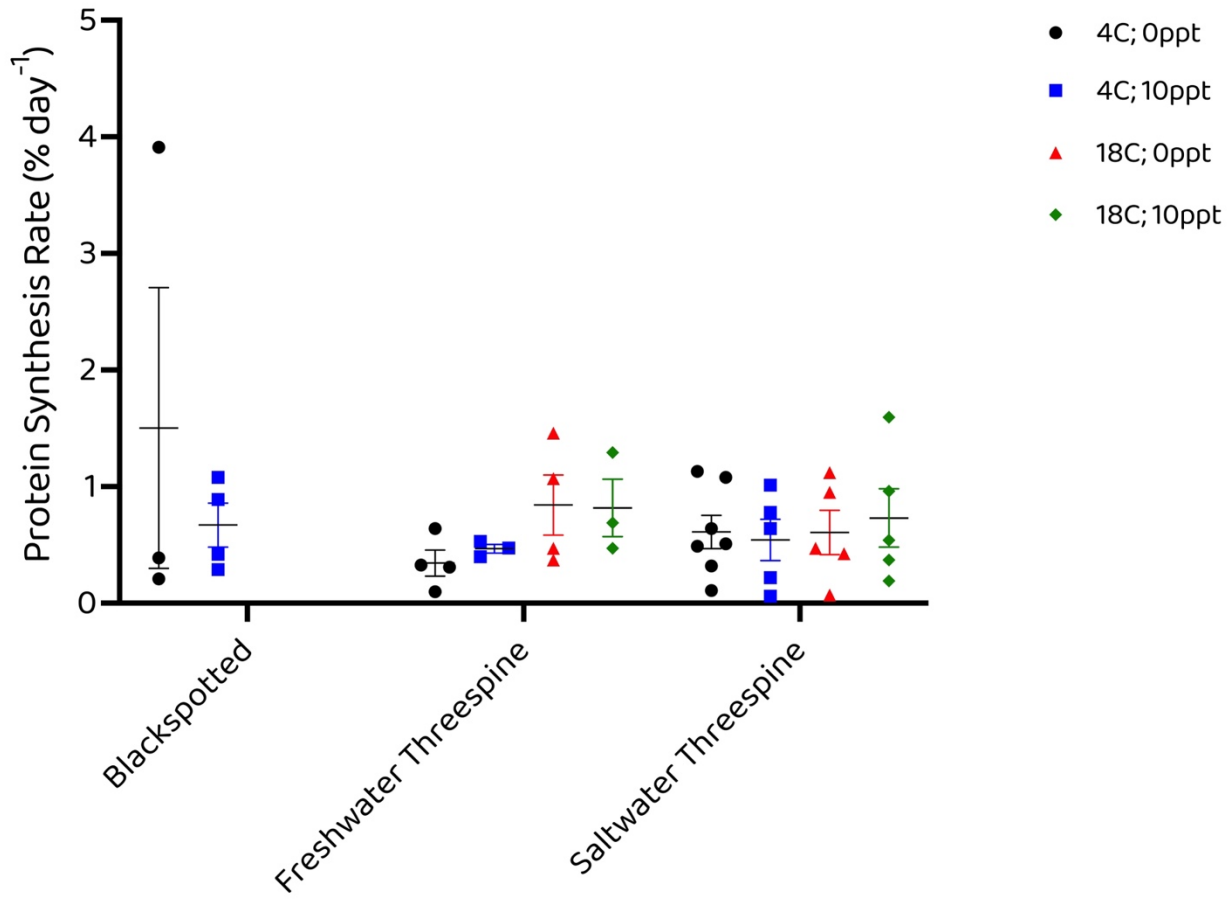

Figure 6. Hepatic protein synthesis rates of blackspotted stickleback (n=3-7 families; 1-2 individuals per family), freshwater (0 ppt)-reared common threespine stickleback (n=6-7 families; 1-4 individuals per family), and saltwater (10 ppt)-reared common threespine stickleback (n=7 families; 1-2 individuals per family) adults in freshwater (0 ppt) and saltwater (10 ppt) at 4°C ('winter' treatment) and 18°C ('spring' treatment). Each data point represents the average rate of D_5-phenylalanine incorporation for an individual family, with the central line representing the mean of all families ±SEM.

3.2.5 Hepatosomatic and Gonadosomatic Indices

Blackspotted stickleback sample sizes were too small to provide sufficient statistical power for accurate analysis of HSI and GSI. Similarly, sex specific sample sizes were too small among saltwater and freshwater threespine sticklebacks across treatments to power a reliable analysis of HSI and GSI. Indeed, HSI and GSI are known to differ between males and females, such that grouping male and female results can be misleading. Raw data from HSI and GSI measurements is shown in supplementary figure 2.

3.2.6 Mortality Rate

Mortality rate during the combined stressor experiment, compared across families, was affected by interactions between species and salinity ($p=0.01$) and between salinity and temperature ($p=0.008$). There was also a main effect of temperature ($p=0.03$). However, there were no differences between species x salinity x temperature groups (Figure 8a). The differences in mortality rates can likely be attributed to tank effects, which were significant ($p<0.0001$; Figure 8b).

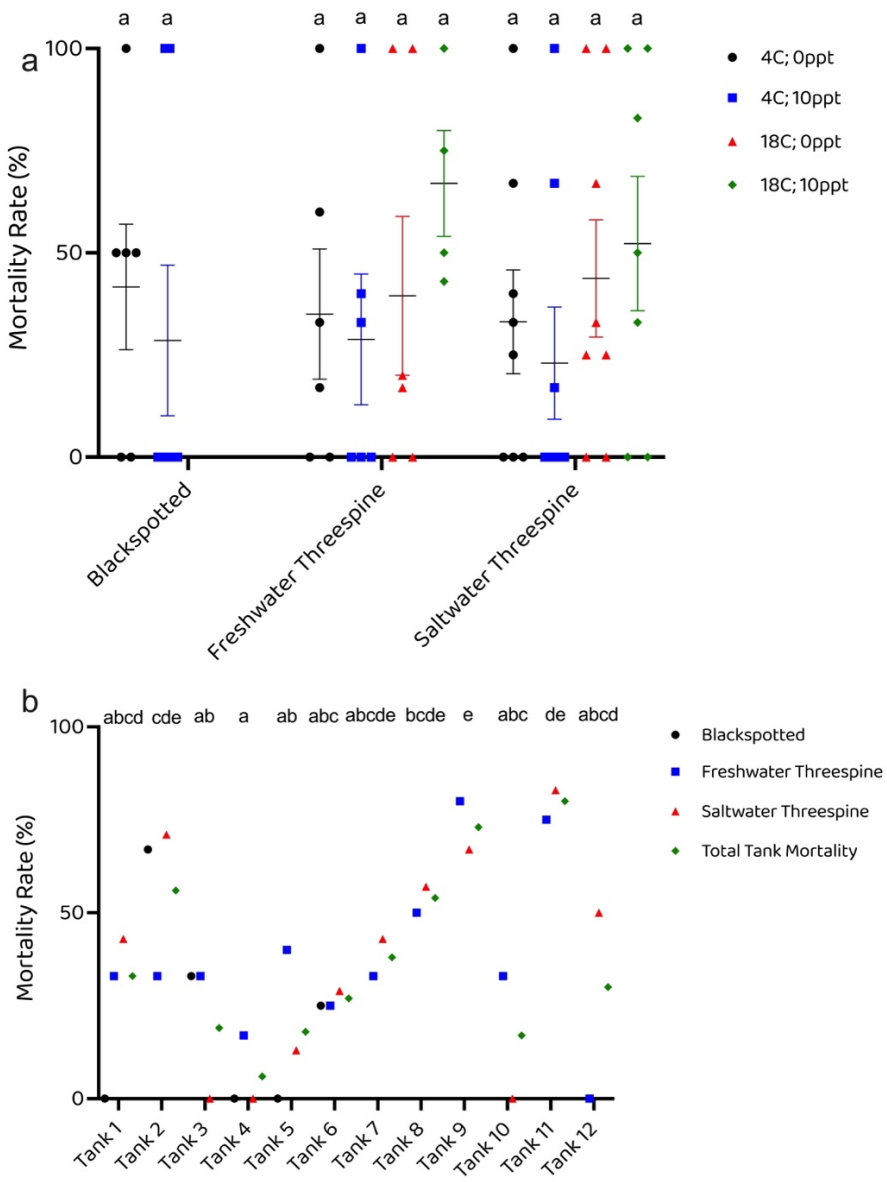

Figure 8. Mortality rates calculated by family (panel a) and by tank (panel b) of blackspotted stickleback, 0 ppt-reared common threespine stickleback, and saltwater 10 ppt-reared common threespine stickleback adults in 0 ppt and 10 ppt at 4°C and 18°C. In panel a, each data point represents the mortality rate within individual families (n = 3-25 individuals per family) with the central line representing the mean of all families ±SEM and, in panel b, each data point represents the mortality rate of each species in each treatment tank.

4. Discussion

The full set of physiological mechanisms required to allow euryhaline fish that normally inhabit brackish and marine habitats to colonize, and live their entire life in, freshwater are not fully understood (but see Shaughnessy and Bystriansky 2024; Shrimpton 2012; Kultz 2015; Ishikawa et al. 2019; Velotta et al. 2022). Stickleback fishes provide a good system in which to study the mechanisms necessary for the successful colonization of freshwater lakes because, within this group, closely related species differ in their extent of freshwater colonization. Furthermore, certain Atlantic Canadian sticklebacks — the blackspotted stickleback (*Gasterosteus wheatlandi*) and the white ecotype of the threespine stickleback (*Gasterosteus aculeatus*) — are freshwater tolerant as adults despite having a saltwater-exclusive distribution in the wild, suggesting that freshwater colonization is limited even when adult salinity tolerance is high, unlike other stickleback (e.g., Fifteenspine and Japanese Sea; Elofsson et al. 2003; Ishikawa et al. 2016). I predicted that freshwater colonizing species, such as the Atlantic Canadian common threespine ecotype (*Gasterosteus aculeatus*) and the fourspine stickleback (*Apeltes quadracus*), have a higher capacity to tolerate freshwater during fertilization and development, and during adulthood when combined with cold temperatures, relative to saltwater restricted species. In this thesis, I took a comparative approach to study freshwater tolerance during early life stages and in cold temperatures in adults.

My results indicate that an inability to reproduce in freshwater may have prevented the blackspotted stickleback, but not the white threespine stickleback, from successfully colonizing freshwater habitats. However, once fertilized, saltwater-restricted and freshwater colonizing Atlantic Canadian sticklebacks were found to be equally capable of tolerating freshwater during early life stages and as adults during cold acclimation. Together these findings suggest that

fertilization can limit successful colonization of freshwater habitats in certain sticklebacks. However, other factors, outside of abiotic tolerance limits, are predicted to also be critical in restricting the range of the blackspotted stickleback to brackish and saltwater environments. For example, biotic factors such as inter-specific competition for breeding habitats (Whoriskey and Fitzgerald 1985) or food quality (e.g., Ishikawa et al. 2019) may have limited blackspotted, and potentially white threespine stickleback, success in freshwater.

4.1 Effect of salinity on fertilization success

Successful colonization of a novel habitat is dependent upon a species' ability to persist in the habitat, which, in turn, is dependent upon an organism's ability to reproduce in that habitat (Lee and Bell 1999). Contrary to expectation, fertilization success of the saltwater-restricted white threespine stickleback was not salinity sensitive, and was similar to the findings for the common threespine stickleback, which can colonize freshwater. These data suggest that white threespine sticklebacks have retained the ability to reproduce in freshwater since diverging from common threespine sticklebacks 10-12,000 years ago (Samuk 2016). By contrast, and in alignment with my prediction, I found that the non-freshwater-colonizing blackspotted stickleback had significantly lower fertilization success in freshwater (0 ppt) than in brackish water (10 ppt), while other species did not. The blackspotted stickleback's brackish water fertilization success rate was approximately 3-fold higher than their average freshwater fertilization success rate, with extensive variation among clutches.

Sperm of the common threespine stickleback is capable of successful external fertilization in both freshwater and saltwater owing to the ovarian fluid, within which the eggs are encased upon release, which creates a micro-environment conducive to fertilization (Le Comber et al. 2004; Alavi and Cosson 2006; Elofsson et al. 2006). Threespine stickleback sperm

is motile for only 30-60 seconds in freshwater, whereas the presence of ovarian fluid facilitates sperm motility for several hours (Elofsson et al. 2006). The protection afforded by ovarian fluid is not universal across euryhaline fishes, however. Various euryhaline fish species, who are capable of fertilizing offspring in both freshwater and saltwater, including the California killifish (*Fundulus parvipinnis*) and white sea herring (*Clupea harengus pallasi*), experience a stark decline of sperm function in freshwater relative to saltwater (Able and Palmer 1988; DiMaggio et al. 2016). Further, freshwater fertilization is impossible for the saltwater-restricted fifteenspine stickleback owing to complete inactivity of sperm in freshwater, regardless of the presence of ovarian fluid (Elofsson et al. 2003). However, my study suggests that freshwater fertilization in the blackspotted stickleback is not solely limited by sperm function, as there was some freshwater fertilization success, suggesting that their sperm is at least marginally functional in freshwater with ovarian fluid. Thus, blackspotted stickleback freshwater fertilization may be limited by viability of the female gametes. Indeed, marine and freshwater fishes vary greatly in how oocytes are prepared for spawning via a number of mechanisms, including the extent of egg hydration regulated by aquaporin deposition (Jorgen et al. 1999; Finn and Cerda 2024; Chen et al. 2022).

In both saltwater and freshwater environments, eggs (i.e., ova) face the full strength of the salinity gradient upon spawning but are unable to actively counter osmosis and diffusion. Thus, during oocyte maturation, some species of fishes can equip eggs with appropriate defences for the environment (Jorgen et al. 1999; Finn and Cerda 2024; Chen et al. 2022). In saltwater, eggs are equipped with a hydration reservoir to compensate for water loss whereas in freshwater, eggs are given a low water content and a less permeable vitelline membrane to inhibit ion loss (Jorgen et al. 1999; Finn and Cerda 2024). In my study, all oocytes were prepared for brackish

water because parents were kept at, and females became gravid in, 10 ppt. The successful freshwater fertilization observed in threespine and white threespine sticklebacks likely owes to the protection of their ovarian fluid (Le Comber et al. 2004; Alavi and Cosson 2006; Elofsson et al. 2006). Among other euryhaline fishes, such as the common killifish (*Fundulus heteroclitus*), saltwater-acclimated individuals cannot produce freshwater-viable eggs upon acute exposure to freshwater (Able and Palmer 1988). Indeed, Able and Palmer (1988) found that such acute freshwater exposure causes osmotic failure of the eggs.

Appropriate salinity preparation of eggs may depend on the ovarian osmolality, which has been shown to change with salinity acclimation in euryhaline species (Palmer and Able 1987). Thus, the blackspotted stickleback's poor freshwater fertilization success may owe to inappropriate oocyte preparation and insufficient protection from the ovarian fluid. Future experiments should acclimate parental fish to freshwater prior to breeding, which is a more ecologically relevant experiment than exposure of gametes without parental acclimation. Indeed, acclimation of both common killifish sexes to freshwater increased freshwater fertilization capacity, and female, but not male, freshwater acclimation was required for fertilization success at 0 ppt, suggesting that the viability of female gametes relies heavily on prior female freshwater acclimation (Palmer and Able, 1987). Palmer and Able (1987) suggest that changes in female ovarian fluid osmolarity that occur with maternal freshwater acclimation plays a critical role in preparing eggs for the hypoosmotic shock of freshwater. Additionally, although my study suggests that saltwater populations of common threespine sticklebacks can successfully fertilize offspring without freshwater acclimation, this is not necessarily true across stickleback populations (Brown et al. 2012; Taugbol et al. 2017). Furthermore, future studies should investigate whether freshwater fertilization is limited by the male or female gametes by crossing

the less-freshwater tolerant blackspotted eggs or sperm with the more freshwater tolerant threespine stickleback eggs or sperm in hybrid crosses split between 0 ppt and 10 ppt. Blackspotted and threespine sticklebacks can successfully cross fertilize, although the resulting embryos are not viable (Hendry et al. 2009). While I do not believe sperm function is the major factor limiting fertilization, sperm function in stickleback can also beneficially acclimate to environmental salinities (Taugbol et al. 2017), so should also be studied.

Lastly, while I could not measure freshwater fertilization success of the fourspine stickleback due to limited sample sizes, this should be measured in the future. Based upon their successful colonization I predict that the marine populations of fourspine stickleback will respond similarly to the common threespine stickleback marine population, wherein fertilization success will not differ between freshwater and saltwater. Understanding how fourspine stickleback fertilization success compares to the common threespine stickleback, and whether they are capable of freshwater fertilization upon acute exposure, would further our insight into whether the convergent evolution of freshwater tolerance between these species utilizes similar or distinct mechanisms.

4.2 Embryonic Stage

4.2.1 Effect of salinity and species on development rate, survival to four days post fertilization, and incubation time

To investigate how stickleback embryos may be affected by freshwater, I measured the rate of embryonic development, embryonic survivorship, and incubation time. I found that the only effect of salinity was a small decline in survivorship of the white threespine stickleback in brackish relative to freshwater, which is likely due to outliers at 10 ppt (Figure 2b). There were

otherwise no differences between salinities for any species. Overall, these results suggest that, once fertilized, the blackspotted, white threespine, common threespine, and fourspine stickleback species are all equally tolerant to freshwater during embryonic development, contrary to my predictions. My results contrast with a host of studies investigating the embryonic salinity tolerance of euryhaline anadromous fish species, who live the majority of their adult lives in saltwater but migrate into freshwater to spawn, which find that embryonic exposure to saltwater induces increased mortality rates (DiMaggio et al. 2016; Winger and Lasier 1994; Morgan et al. 1992; Brown et al. 2012; Wagner 1967). Similarly, estuarine killifish, who can tolerate freshwater as adults, experience extreme reductions in their successful hatch rates when embryos are exposed to freshwater (Tay and Garside 1974; Brown et al. 2012). In contrast, Atlantic Canadian stickleback embryos are unaffected by freshwater, demonstrating an exceptional embryonic capacity to tolerate freshwater. However, survival alone does not provide a complete gauge of environmental tolerance. To truly understand how well an organism is coping with their environment, a metric for the ability to produce and use energy, such as metabolic rate, is required. This is because it is possible for organisms to perform well amid physiological stress by metabolically compensating or potentially re-allocating energetic resources (Auer et al. 2015; Metcalfe and Monaghan 2001).

4.2.2 Effect of salinity and species on embryo metabolic rate

During exposure to stressful environments, a fish may require more energy to survive, increasing metabolism. This elevated metabolic cost is reflected in a high standard or routine metabolic rate, normally estimated by measuring oxygen consumption (Auer et al. 2015). Such increases in metabolic rate will decrease the aerobic scope (maximal minus standard metabolic rate), leaving less remaining energy to fuel other processes, such as growth and development

(Norin and Metcalfe 2019). If the additional energetic requirements associated with maintaining homeostasis during stressful situations cannot be met by available energetic resources and physiological capacities, then trade-offs among functions occur. Such trade-offs often result in decreased growth rates and activity levels, potentially reducing organismal fitness (Norin and Metcalfe 2019). Alternatively, in some species resting metabolic rates decrease in stressful environments and biological processes slow down until conditions improve. This decreased rate prioritizes basic biological functions and does not allocate energy to fuel activities such as growth and reproduction (Norin and Metcalfe 2019). However, contrary to expectation, I found no evidence of an increased metabolic cost of development in freshwater compared to in 10 ppt water among saltwater-restricted blackspotted sticklebacks. Combined with my findings that blackspotted embryos at 0 ppt and 10 ppt did not differ in development times or hatch rates, these data suggest they are equally freshwater tolerant as other stickleback species at the embryonic stage.

Interestingly, steelhead salmon, a euryhaline anadromous salmonid, also displays similar embryonic metabolic rates in freshwater and saltwater, despite having salinity-dependent survivorship (Morgan et al. 1992). However, the metabolic rate of steelhead salmon became salinity dependent following hatch and larvae failed to develop normally in salinities exceeding 8 ppt (Morgan et al. 1992). Euryhaline freshwater tilapia (*Oreochromis* sp.) embryos do not change their metabolic rate when acutely exposed to salinities up to 12 ppt but increase their metabolic rate within one hour of exposure to 16 ppt and 20 ppt, the lethal salinity (Rahmah et al. 2020); after 30 days of acclimation to 16 ppt, surviving embryos decreased their metabolic rates to match the resting rate observed in freshwater, suggesting acclimation had occurred. However, while this metabolic acclimation suggests an ability to cope with 16 ppt, the acclimation period

was accompanied by a 60% mortality rate, indicating that embryonic tilapia have low tolerance to increased salinity (Rahmah et al. 2020). Similarly, Fridman et al. (2012) found that euryhaline Nile tilapia (*Oreochromis niloticus*) embryos experience significant declines in survivorship and hatching success and impeded embryonic development when incubated in salinities over 7.5 ppt, and yet there were no salinity-induced differences in metabolic rates until the larval stage at 3 days post hatch. My study found that salinity did not affect survival, development, size, or metabolic rate among embryonic Atlantic Canadian sticklebacks. However, even in cases where embryonic tolerance and survival are negatively affected by salinity, metabolic rates do not seem to reflect these high mortality rates (Morgan et al. 1992; Rahmah et al. 2020; Fridman et al. 2012). These data indicate that embryonic metabolic rate may not be a reliable proxy for embryonic salinity tolerance in salinity sensitive euryhaline fishes. Rather, survivorship, incubation duration, embryo osmolality, or yolk sac usage may be better metrics for embryonic salinity tolerance (DiMaggio et al. 2016; Machado and Podrabsky 2007; Romney et al. 2019).

4.3 Adult Freshwater Tolerance: Combined Stress of Freshwater and Cold Temperature

I predicted that saltwater-exclusive species, the blackspotted stickleback and white threespine stickleback, would be more vulnerable than freshwater colonizing species (i.e., threespine and fourspine stickleback) to the combined stress of cold temperatures and freshwater. Upon reaching adulthood, fish in freshwater lakes encounter winter temperatures that exacerbate the existing challenge of ionoregulation (Morash et al. 2021; Volkoff and Ronnestad 2020; Buhariwalla et al. 2012) by altering the state of the cellular membranes, across which ions are transported, by making the fluid-like state of the lipid bilayer more solid and impeding transport (Buhariwalla et al. 2012). I predicted that freshwater combined with cold temperature poses a

physiological obstacle to non-freshwater-colonizing species. While cold temperatures decreased stickleback metabolic rates, as predicted (Hurst 2007; Handeland et al. 2008; Reid et al. 2022l Gibbons et al. 2017; Middleton et al. 2024), I found that the standard and active metabolic rates did not differ between species or salinities, suggesting that limitations in aerobic scope are not a key mechanism limiting colonization of blackspotted stickleback.

Ressel et al. (2022) found that standard metabolic rates of common threespine stickleback increase with treatment temperature, but the aerobic scope is not conserved across temperatures because the maximal metabolic rate was disproportionately lower under winter temperatures (5°C) than under summer temperatures (20°C). Thus, this study suggests that cold temperatures impose limitations on the aerobic scope (Ressel et al. 2022). In contrast, Bruneaux et al. (2014) compared metabolic performances of coastal and freshwater ninespine stickleback (*Pungitius pungitius*) populations acclimated to various temperatures across a winter to summer thermal spectrum and found conservation of the aerobic scope across temperatures (6°C, 11°C, 19°C). Thus, seasonal shifts of the aerobic scope appear to vary among freshwater colonizing stickleback species. My results suggest that the upper metabolic limit does not vary across seasonal temperatures or salinity in the threespine stickleback, nor across salinity in the blackspotted stickleback, and, thus, indicate aerobic scope conservation under the conditions tested and high tolerance in both species. However, my active metabolic rates were low, likely due to poor species-specific suitability of the methods, which may be reflected in the upper metabolic limit.

I attempted to measure the maximal metabolic rate in my experiments using the standard chase protocol (Norin and Metcalfe 2019; Norin and Clark 2015). Previous work, using swim tunnel respirometry, has consistently shown that the threespine stickleback's maximum

metabolic rate is ~3 times the standard metabolic rate (i.e., a factorial aerobic scope of ~3) (Ressel et al. 2022; Cominassi et al. 2022; Dalziel et al. 2012). However, despite chasing the fish to apparent exhaustion, characterized by cessation of response to stimuli and failure to maintain position in the water column (i.e., fish stopped swimming and lost upright position in favour of rolling with the current in the bucket), I found that threespine and blackspotted sticklebacks stop responding to stimuli far before reaching their maximum metabolic rate, suggesting that exhaustive chase may not be the ideal method for estimating maximal metabolic rate for sticklebacks. The post-chase metabolic rates I collected ranged from two times the standard metabolic rate for the highest measurements, to equal to or slightly below the standard metabolic rate for the lowest measurements. Thus, I termed this data as "active" metabolic rates and could not use it to calculate an accurate aerobic scope. Active metabolic rates and the duration that each fish was chased before reaching apparent exhaustion did not differ between species, salinity, or temperature. Such lack of treatment response suggests that neither salinity nor temperature impact the amount of energy that either stickleback species chooses to expend. Furthermore, comparison of species' mortality rates throughout the adult temperature x salinity experiment reveals that survivorship is also unaffected by species, salinity, or temperature. Indeed, my metabolic rate measurements indicate that the freshwater colonizing common threespine stickleback has no energetic advantage over the non-freshwater colonizing blackspotted stickleback under any of the environmental treatments.

Although I was not able to calculate a reliable aerobic scope, some of the main allocations of the aerobic scope are growth and reproduction (Wootton 2011; Madenjian et al. 2024), so I measured these traits to see get a sense of potential functional limitations on energy expenditure. As expected, growth rates, as calculated by both mass and length, were lower under

the cold temperature than the warm temperature (Hurst 2007; Handeland et al. 2008; Reid et al. 2022; Gibbons et al. 2017; Middleton et al. 2024; Lefebure et al. 2011). Furthermore, there was an overall species effect on growth rate assessed by standard length, but not by mass, such that the blackspotted stickleback had a significantly lower growth rate than the saltwater threespine. I also investigated hepatic protein synthesis rates as a potential mechanism underlying growth differences. Contrary to expectation, hepatic protein synthesis rates did not differ across treatments, suggesting neither temperature, salinity, nor species elicits a greater response in hepatic protein. Indeed, similar to as in my study, Middleton et al. (2024) also found that hepatic protein synthesis in arctic-dwelling brook char (*Salvelinus fontinalis*) does not differ between temperatures (8°C and 2°C).

Lastly, I quantified hepatosomatic and gonadosomatic indices as indications of potential energetic stores and readiness to spawn (Chellappa et al. 1995). Hepatosomatic and gonadosomatic indices correlate with energetic capacity, such that a high index correlates with large lipid, protein, glycogen, and total energetic reserves (Chellappa et al. 1995). Fish within my study were fed ad libitum, leaving freshwater (and freshwater-cold temperature combined stress) stress as the only variable(s) that may impact energetic reserves and, subsequently, hepatosomatic and gonadosomatic indices. Although my study did not have sufficient sample sizes to test if these traits varied among species and salinities, the data trends suggest a lack of freshwater induced impact on either index. Indeed, the hepatosomatic index appears to be higher at 4°C relative to 18°C, a pattern that has also been observed among Arctic ninespine sticklebacks (Bruneaux et al. 2014). Cold-induced elevation of the hepatosomatic index likely reflects a cold stress coping strategy, wherein accumulation of energetic reserves supports gluconeogenesis and lipid metabolism. Further, the hepatosomatic index appears similar between

salinity treatments within each temperature regime. The gonadosomatic index does not appear to differ between temperature or salinity treatments. My observation of hepatosomatic and gonadosomatic index similarity across groups, with overall temperature effects, is consistent with the pattern observed in metabolic capacity, growth, and protein synthesis rates suggesting all adult stickleback species tested are able to tolerate exposure to cold freshwater equally well.

In my study, all fish were fed to satiation with the same nutritionally balanced diet, rich in omega fatty acids, including docosahexaenoic acid (DHA). This leaves a gap in our understanding of how the blackspotted stickleback would cope with freshwater given a more ecologically relevant diet. Northern latitude freshwater habitats are characterized by variability in food availability and diets lower in essential fatty acid content, particularly deficient in DHA (Morash et al. 2021; Ishikawa et al. 2019; Kainz et al. 2009). Dietary DHA is chiefly sourced from marine ecosystems, where it is produced by certain primary producers, and transported into freshwater and terrestrial systems via consumption of DHA-rich primary and secondary consumers, such as krill, mollusks, and fish (Gladyshev et al. 2013). DHA travels efficiently up the food chain, accumulating in the body mass of higher trophic levels, such that fish, and especially marine zooplanktivorous fish, boast the highest content of DHA (Ferreira et al. 2022; Gladyshev et al. 2013). Comparing the prey of anadromous (marine diet) and stream-resident (freshwater diet) populations of dolly varden trout (*Salvelinus malma*) provides a clear example of how dietary DHA content differs between marine and freshwater habitats. Freshwater-confined individuals consume diets consisting predominantly of insects, which may consume DHA-synthesizing microalgae that contain little DHA, supplemented by other fish, amphipods, and roundworms (Stewart et al. 2009; Gladyshev et al. 2013). By contrast, anadromous individuals consume other fish as the bulk of their diet and a wide variety of DHA-rich mollusks

and zooplankton (Stewart et al. 2009). As a result of the dietary differences between marine and freshwater environments, wherein marine diets are more diverse and nutritious, anadromous trout grow larger than stream residents (Stewart et al. 2009). This pattern persists among other anadromous and non-anadromous salmonid populations, including the Pacific salmon (*Oncorhynchus nerka*; Kaeriyama et al. 1995; Buktenica and Larson 2009). Diet may play a critical role in local adaptation among sticklebacks (Day et al. 1994), and the nutritional limitations inherent with freshwater habitats may, in fact, limit colonization potential of the blackspotted stickleback. Like many marine fishes, blackspotted sticklebacks have only one copy of *Fads2*, limiting their capacity for endogenous DHA biosynthesis (Ishikawa et al. 2019). In addition to inhibiting their nourishment, a single *Fads2* copy may further limit the blackspotted stickleback's capacity for homeoviscous adaptation to the cold and, thus, inhibit their ability to successfully overwinter (Snyder and Hennessey 2004). In my study, all fish were fed a DHA rich diet and, therefore, the impact of *Fads2* copy number on freshwater tolerance when dietary DHA is deficient cannot be inferred from my results. However, this would be an important avenue to explore in future work.

Another limitation of my study was a limited sample size of blackspotted sticklebacks, such that it was not possible to have a summer (18°C) control for the species in my tests of temperature and salinity effects (section 2.3.1). It would have been valuable to compare their physiological parameters between winter temperatures and summer temperatures and, additionally, to compare growth and maturation between warm freshwater and warm saltwater. Additionally, I saw high variability among families, which may, in part, be due to small sample sizes, which were as low as three families within a treatment. Members within a population may have considerable genetic variation in metabolism (Burton et al. 2011; Johnson et al. 2016),

which may account for the observed variability in blackspotted stickleback's freshwater tolerance capacities. Under summer conditions (18°C, 0 ppt and 10 ppt), towards the end of the experiment, common threespine stickleback males began to exhibit breeding colours while females became gravid. It is unknown whether blackspotted sticklebacks are capable of achieving breeding condition in freshwater and I could not test this due to sample size and time limitations. Furthermore, maternal nutrition has been linked to offspring success (Perez-Atehortua et al. 2023), providing further motive to study the freshwater tolerance capacity of blackspotted sticklebacks under realistic nutritional circumstances. If blackspotted sticklebacks cannot obtain proper nutrition in freshwater then their offspring may not be viable, if they are able to reproduce in freshwater at all.

4.4. Perspectives and Conclusions

Overall, the results of my study show that fertilization in freshwater might limit freshwater colonization in blackspotted stickleback, but all traits measured at subsequent life history stages do not differ between this species and the successfully colonizing threespine stickleback. Although this is the first study to investigate the blackspotted stickleback's capacity to cope with freshwater as embryos and as adults under winter temperatures, the blackspotted stickleback's ability to tolerate chronic freshwater exposure is consistent with previous work demonstrating their high adult and juvenile tolerance to warm freshwater (Bernhardsson 2021; Campeau et al. 1984). Campeau et al. (1984) showed that both blackspotted and threespine sticklebacks struggle to cope with freshwater as juveniles. Though my study did not investigate the juvenile stage specifically, I had high mortality rates of freshwater-reared blackspotted sticklebacks during this stage; Campeau et al. (1984) observed stunted development and lethargy of juvenile blackspotted stickleback in 0 ppt. Together, these data may suggest that blackspotted

sticklebacks struggle most in freshwater during the juvenile stage. However, Campeau et al.'s (1984) study differed from mine as individuals studied were: 1) acutely exposed during the juvenile stage; and 2) sampled from marine populations. Thus, it would be interesting to explore: 1) whether threespine stickleback juveniles from my populations show similar freshwater sensitivity; and 2) if juveniles from a freshwater-adapted population fare better in freshwater relative to those from marine populations.

Together, the similar freshwater tolerance capacities of Atlantic Canadian marine threespine and blackspotted stickleback populations found by Campeau et al. (1984) and my research indicate both species have a similar capacity to tolerate freshwater, suggesting that freshwater tolerance as juveniles and adults is not the factor limiting freshwater colonization. Rather, a basic ability to tolerate freshwater may be a prerequisite for freshwater colonization; a prerequisite that both threespine and blackspotted sticklebacks possess. However, further improvements to freshwater tolerance have been detected by Gibbons et al. (2017a), wherein freshwater populations have evolved to increase growth in winter temperatures (4°C) and use a different combination of genes for gill transmembrane ion transport and epithelial structure than marine populations (Gibbons et al. 2017b). These differences in lake populations of Pacific threespine stickleback are hypothesized to be due to selection following colonization > 10,000 years ago. Indeed, Bruneaux et al. (2014) also found that Baltic freshwater populations of ninespine stickleback have higher maximal metabolic rates and, consequently, larger aerobic scopes at winter temperatures relative to marine populations.

Such local adaptation in salinity or temperature tolerance has also been found in other fishes. For example, Shultz and Conover (1997) studied genetically distinct populations of estuarine Atlantic silverside (*Menidia menidia*) who differ in overwintering survivability. They

attributed the variation in winter mortality to genetically based differences in the rate of accumulation of energetic reserves (Shultz and Conover 1997). Analogously, the physiological mechanisms underlying salinity tolerance differ between anadromous and landlocked populations of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*), such that landlocked populations have evolved reduced saltwater tolerance (McCormick et al. 2019; Staurnes et al. 1992). Gibbons et al. (2016) also found that the Pacific marine population fared better in saltwater than in freshwater, while my study found no difference between Atlantic Canadian threespines' freshwater tolerance, suggesting that Atlantic marine threespine sticklebacks have a more robust freshwater tolerance than Pacific populations and may better tolerate freshwater colonization.

I also tested the potential role of early life freshwater acclimation on salinity and temperature tolerance in adults and found no difference between the freshwater tolerance capacities of saltwater and freshwater reared threespine sticklebacks, suggesting that developmental plasticity is likely not a major factor influencing adult tolerance. However, the lack of variation in tolerance within an acclimation treatment makes it hard to evaluate the role for developmental plasticity, and it would be interesting to test the potential effects of multigenerational plasticity (Massey and Dalziel 2023; Pilakouta et al. 2019).

Lastly, my study ended with quantifying freshwater tolerance amid cold temperatures in an effort to assess the severity of a simulated-winter ionoregulatory challenge. Consequently, my study did not continue into the adult spring stage (i.e., breeding season), leaving a remaining knowledge gap as to how the blackspotted and common threespine sticklebacks differ in the next step of their life history: reproduction. The ability to reproduce in freshwater is a key criterion to colonizing freshwater habitats (Lee and Bell 1999) and I found poor *in vitro* freshwater

fertilization rates of 10 ppt acclimated blackspotted stickleback parents. It remains to be seen if blackspotted sticklebacks are capable of freshwater reproduction after being acclimated to freshwater, either over their full lifetime (developmental plasticity) or as adults reared in freshwater. For example, can this species acclimate to improve their ability for freshwater reproduction (Palmer and Able 1988)? Would acclimating blackspotted stickleback males to freshwater improve fertilization success (i.e., as in saltwater; Taugbol et al. 2017)? And can adult blackspotted stickleback females become gravid in freshwater? Answering these questions will help to further assess the role of low salinities in limiting freshwater colonization in sticklebacks.

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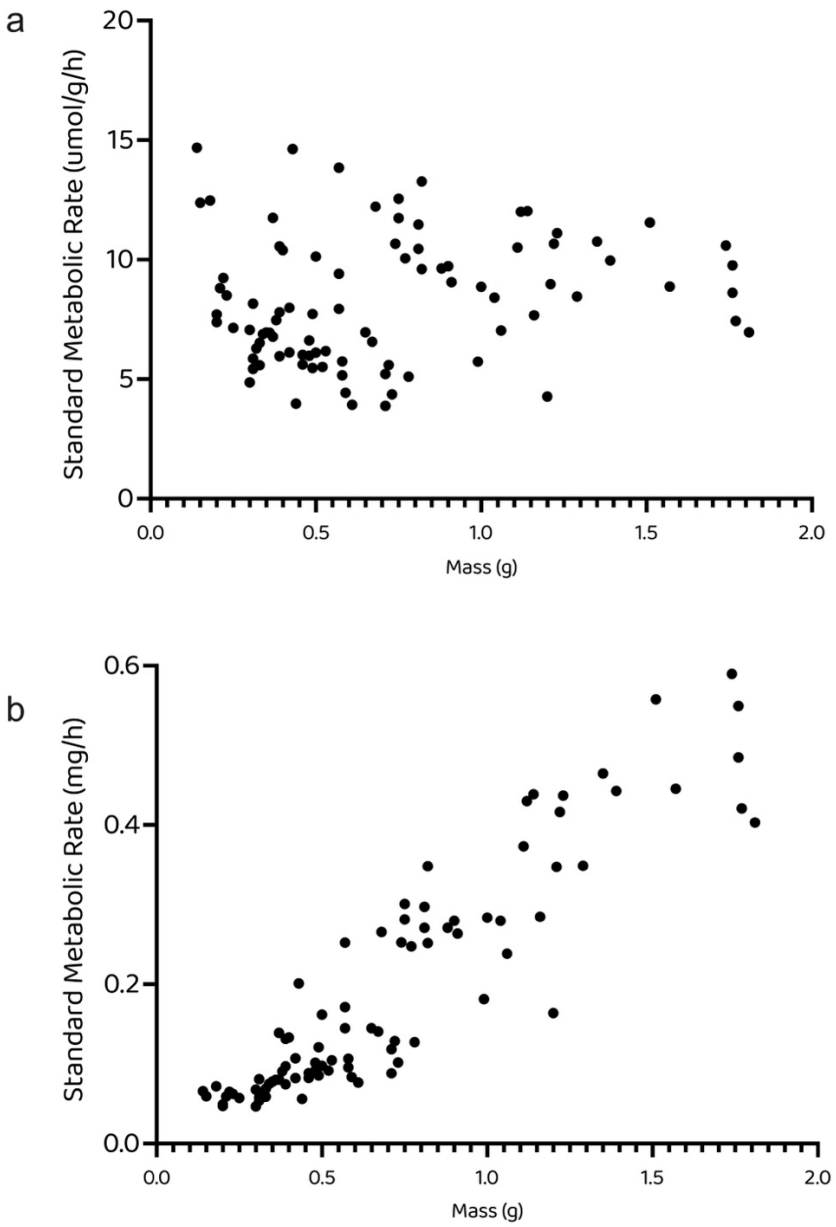
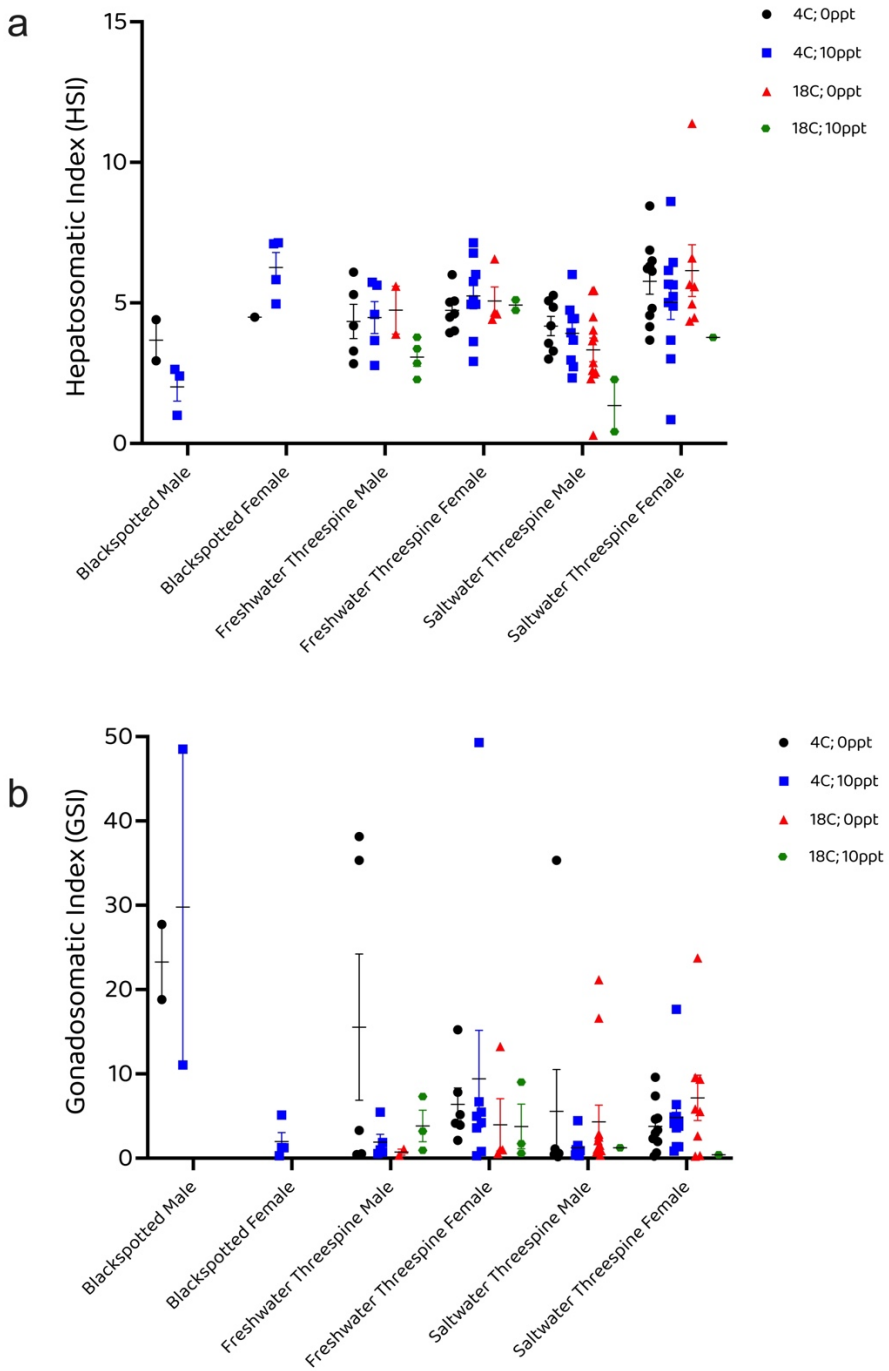
Supplementary Material



Supplementary Figure 1. Linear regression of mass standardized oxygen consumption rates (μmol g^{-1} h^{-1}; R^2= 0.03; panel a) and non-standardized oxygen consumption rates (μmol h^{-1}; R^2= 0.84; panel b) for all species (n= 89 individuals) across treatments (4°C, 0ppt; 4°C, 10ppt; 18°C, 0ppt; 18°C, 10ppt). There is no significant effect of mass on size-corrected metabolic rates, suggesting isometric effects of mass in this study ($F_{1,87}$=2.89; p=0.09). Each data point represents an individual's oxygen consumption rate.

Hepatosomatic (HSI) and gonadosomatic (GSI) indices were calculated at the end of the exposure, to reflect body condition and readiness to spawn (Rizzo and Bazzoli 2020).



Supplementary Figure 2. Sex-specific hepatosomatic index (HSI; panel a) and gonadosomatic index (GSI; panel b) of blackspotted stickleback (n= 1-4 individuals per sex), freshwater threespine stickleback (n= 2-9 individuals per sex), and saltwater threespine stickleback (n= 1-12 individuals per sex) adults in freshwater (0 ppt) and saltwater (10 ppt) at 4°C ('winter' treatment)

and 18°C (‘spring’ treatment). Each data point represents the HSI or GSI of an individual, the central line represents the mean of all individuals within the category ±SEM.