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

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ABSTRACT

Colonization of freshwater habitats from marine environments exposes organisms to novel combinations of temperature and salinity, but little is known about physiological responses to the interactive effects of these stressors. Here, we examined the effects of temperature (14 versus 4 °C) and salinity (11 versus 0.3 ppt) on gill gene expression in marine, anadromous, and freshwater populations of threespine stickleback (*Gasterosteus aculeatus*). Expression of the epithelial calcium channel was not affected by temperature or salinity, but had significantly higher expression in the freshwater ecotype. The combination of low temperature and low salinity had non-additive effects on the expression of the Na^+/H^+ exchanger. Fish exposed to the combination of low temperature and low salinity had expression levels similar to fish exposed to either factor in isolation. Expression of Na^+,K^+-ATPase α-subunit was greater in fish exposed to low temperature and low salinity than in fish exposed to the factors separately, and this effect was the most pronounced in the marine ecotype. We also examined the interactive effects of salinity and temperature on gill morphology in the marine ecotype, and observed non-additive effects. Low temperature increased the size of the interlamellar cell mass in fish held at 11 ppt, but not at 0.3 ppt, and the effect of low salinity was in the opposite direction in fish at high and low temperatures. These data demonstrate interactive effects of temperature and salinity and highlight that overwintering in cold freshwater was likely a physiological challenge for marine stickleback as they colonized freshwater following the last glaciation.

1. Introduction

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

The threespine stickleback (*Gasterosteus aculeatus*) is one of many organisms that have been able to overcome the barriers presented by the transition between freshwater and saltwater habitats (Lee and Bell, 1999), successfully colonizing numerous freshwater habitats in the Northern Hemisphere. In stickleback, this colonization has been associated with repeated adaptation and parallel evolution of a variety of traits since marine stickleback colonized freshwater habitats 10,000–20,000 years ago following the recession of Pleistocene glaciers (Baker, 1994; Bell and Foster, 1994; Boughman, 2007; Colosimo et al., 2005; Jones et al., 2012a, 2012b; McKinnon et al., 2004; McKinnon and Rundle, 2002; McPhail, 1994; Schluter, 2009). There are many abiotic and biotic factors that differ between marine and freshwater habitats, but two key abiotic factors that may play a role in transitions to year-round freshwater residency are salinity and temperature. The change in salinity between these habitats is an obvious potential driver of evolutionary change in physiological processes, given the substantially

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different osmoregulatory physiologies exhibited by fishes in marine and freshwater environments (Evans et al., 2005), but temperature changes between the habitats may also play an important role. For example, it has been hypothesized that the ancestral stickleback that colonized fresh water may have been anadromous (Colosimo et al., 2005; Haglund et al., 1992; Kitano et al., 2012; McKinnon et al., 2004; Orti et al., 1994; Taylor and McPhail, 1999, 2000), and thus would have been able to tolerate changes in salinity. However, in British Columbia, anadromous stickleback return to the ocean before the winter, and therefore do not experience the cold winter temperatures that occur in fresh water (Hagen, 1967). Adoption of year-round freshwater residency in the north-temperate zone therefore requires the ability to survive and overwinter in the novel combination of low salinity and low temperature. Recent work on the euryhaline Atlantic killifish (*Fundulus heteroclitus*) (Buhariwalla et al., 2012) and other anadromous species (McCormick et al., 1997; Stanley and Colby, 1971) has shown that the combination of low salinity and low temperature may be particularly challenging for fish ionoregulation by causing a decreased capacity to maintain plasma ion levels. This suggests that the combination of these two abiotic factors may have posed a significant challenge to colonization of freshwater habitats in the north-temperate zone.

Although many studies have examined osmoregulatory physiology in freshwater and marine populations of fish (Brennan et al., 2015; Kozak et al., 2014; Velotta et al., 2014, 2015; Whitehead et al., 2011), including stickleback (DeFaveri et al., 2011; Divino et al., 2016; Hasan et al., 2017; Jones et al., 2012a, 2012b; Kusakabe et al., 2017; McCairns and Bernatchez, 2010; Rind et al., 2017; Shimada et al., 2011; Taugbøl et al., 2014; Wang et al., 2014), only a single study has addressed the combined effects of both low salinity and low temperature on ionoregulation in stickleback (Schaarschmidt et al., 1999). These authors examined brackish water and freshwater populations of stickleback acclimated to fresh water and brackish water under warm and cold conditions. There were only minor differences in the activity of a few genes involved in ionoregulation between stickleback native to either brackish water or freshwater habitats, yet the combination of low salinity and low temperature resulted in high mortality in stickleback from brackish water habitats (Schaarschmidt et al., 1999). This differential mortality provides support for the idea that the combination of low salinity and low temperature may have posed a challenge to colonization of fresh water by stickleback from the marine environment.

In addition to the osmoregulatory functions carried out by specific ion transporters located in fish gills (Evans et al., 2005; Hwang et al., 2011), the morphology of the gill itself can have major physiological impacts for fish. The gill lamellae are the major structural sites of ion flux and oxygen uptake, and alterations in lamellar surface area directly impact these processes (Nilsson et al., 2012). In addition to modifying lamellar surface area by alterations in blood perfusion (Nilsson et al., 2012), it has been shown that changes in the size of an interlamellar cell mass (ILCM) directly modify lamellar surface area (Sollid et al., 2003). In response to hypoxia (Sollid et al., 2003), warm water (Barnes et al., 2014; Mitrovic and Perry, 2009; Sollid et al., 2005), and exercise (Brauner et al., 2011; Fu et al., 2011; Perry et al., 2012), some fish species are able to remodel their gills by reducing the size of the ILCM. This decrease in ILCM increases lamellar surface area, and is likely beneficial for the increased oxygen uptake required in response to these factors (Nilsson et al., 2012). Additionally, the opposite may be true – when oxygen demand is low, increasing the size of the ILCM may limit energy expended on osmoregulation (the "osmorespiratory compromise"; but see caveats in Nilsson et al. (2012)), and one study in the mangrove killifish *Kryptolebias marmoratus* has shown that salinity changes also elicit changes in ILCM size (LeBlanc et al., 2010).

Here we examined the effects of the combination of low salinity and low temperature on gill ion transporter gene expression in marine, anadromous, and freshwater populations of stickleback, and also examined the effects of salinity and temperature on the ILCM in the marine ecotype. The combined effects of low salinity and low temperature are likely to have posed a challenge to colonization of freshwater habitats from the ocean following glacial retreat, and thus may have driven adaptive evolution in gill functional traits. If ancestral plasticity (in the marine ecotype) represents an adaptive response to exposure to cold fresh water, then we would predict that freshwater-resident stickleback would have been under positive selection for increased plasticity, and thus demonstrate the greatest change in gene expression when exposed to changes in both salinity and temperature, whereas anadromous stickleback, which do not experience fresh water in the winter, would lack the temperature-induced component of this response, and marine stickleback would show the smallest response to both of these factors. Alternatively, if plasticity in the ancestral marine stickleback was maladaptive, we would expect this trait to have experienced negative selection following colonization, and thus we would expect the greatest plasticity in the marine ecotype and the least in the freshwater ecotype. Thus, observed differences in plasticity among these groups can allow us to develop hypotheses about the adaptive value of the observed gene expression plasticity.

2. Materials and methods

2.1. Stickleback populations, acclimation conditions, & time course

Adult stickleback were collected from three populations in British Columbia, Canada in June and July of 2013. Marine (Oyster Lagoon (49°36′43.53″N, 124°01′52.12″W)), anadromous (from the mouth of the Little Campbell River (49°00′52″N, 122°45′33″W)), and freshwater (Trout Lake (49°30′29″N, 123°52′29″W)) stickleback were transported to the laboratory at the University of British Columbia and housed in 100 L glass aquaria with recirculating filtered water. All experimentation and fish husbandry were performed in compliance with the Canadian Council of Animal Care, with an approved animal care protocol (A10-0285). Oyster Lagoon (marine) and Little Campbell River (anadromous) stickleback were initially acclimated to a salinity of 20 ± 0.5 ppt (with Instant Ocean® sea salt), while Trout Lake (freshwater) stickleback were acclimated to 2 ± 0.2 ppt, which are similar to the salinities at their collection locations. All fish were acclimated to a water temperature of 14 °C at a photoperiod of 12 L:12D. Fish were held at a density of 25 fish per aquarium, and were fed bloodworms once daily. We collected 200 marine stickleback and 200 anadromous stickleback (eight aquaria each), but we were only able to collect 100 freshwater stickleback (four aquaria). All fish were acclimated to these conditions for at least 27 and not > 62 days; at which point the acclimation salinities of all fish were changed to 11 ppt (temperature and photoperiod were not altered). Salinity changes were performed gradually (over two to three days), and all fish acclimated in these conditions (14 °C and 11 ppt) for 32–33 days prior to experimental acclimations.

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

At 31 days after the salinity/temperature changes, fish from all four groups were euthanized with an anesthetic overdose (with MS-222 at a concentration of 0.5 g/L, buffered to a pH of 7.0–7.5 with sodium bicarbonate (0.5-1 g/L)). Immediately after euthanasia, fish were weighed, standard length was measured, and the right and left gill baskets were excised. The right gill basket was snap-frozen in liquid N_2 and stored at −80 °C (for RNA extraction), while the left gill basket (from the marine ecotype only) was preserved for microscopy. For microscopy, left gill baskets were immediately placed in Karnovsky's Fixative following excision and stored at 4 °C. After 24 h in Karnovsky's