Ionoregulation in the Cold: A Possible Barrier to Freshwater Colonization?

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Abstract

To test if ionoregulation in winter cold is a barrier to freshwater colonization at high latitudes in fishes, I investigated the ionoregulatory responses of euryhaline marine-adapted Mummichogs (*Fundulus heteroclitus*) and freshwater-adapted Banded killifish (*Fundulus diaphanus*) held in freshwater at 14°C and 0, 4, 14, and 28 days of exposure to winter temperature of 4°C. I predicted that the Banded killifish would be able to maintain their ionoregulation better then Mummichogs in the cold freshwater. I measured maximal activities of gill Na^+-K^+-ATPase and H^+-ATPase, two key ionoregulatory enzymes, and plasma chloride concentrations as a measure of osmotic status. There were no significant differences between species and no effect of cold acclimation on plasma chloride or enzyme activities. The lack of ionoregulatory perturbation by winter cold in either species, especially the seawater-preferring mummichog, do not support the hypothesis that ionoregulation in winter is a barrier to freshwater colonization in fishes.

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Introduction

Ion regulation is a central challenge for fish. Freshwater and saltwater bony fishes (teleosts) are osmoregulators and ionoregulators that maintain internal osmolarities and ion levels (300-400 mOsm/L; primarily Na^+ and Cl^-, 80-140 mmol/L each) that are substantially different than their respective environment. In freshwater, which is very dilute (~0.5 mOsm/L), teleosts must cope with diffusive loss of ions and water gain. Conversely, in seawater, which has a high osmolarity (~1100 mOsm/L) and NaCl (~475 mmol/L) concentration, they must cope with diffusive gain of ions.

To regulate osmolarity and ion levels, teleosts rely upon mitochondrial rich (MR) chloride cells in the gills to take in or excrete sodium and chloride in order to balance that lost or gained, respectively, from passive ion leak between the animal and its environment (Hwang & Lee, 2007). The Na^+-K^+-ATPase pump is an enzyme found on the basolateral (blood-facing) surface of MR cells and plays an important role in ionoregulation in both freshwater and seawater fishes. In seawater, the Na^+-K^+-ATPase generates an electrochemical gradient that causes Cl^- to move from the blood into the MR cells and then across the apical membrane (water-facing) into the water. Na^+ moves through paracellular junctions, following the electrical gradient established by the Cl^- movement (Marshall, 2002). Freshwater fish use Na^+-K^+-ATPase as well as a H^+-ATPase to power active uptake of Na^+ and Cl^- ions from the environment to the blood through the gills, in exchange for H^+ and $HCO3^-$ respectively. The H^+-ATPase, another enzyme found on the surface of MR cells, actively pumps out H^+ to

create an electrical gradient which allows the cells to take in Na^+ (Marshall, 2002). The Na^+ ions are then pumped out of the MR cells into the blood stream using the Na^+-K^+-ATPase, and Cl^- follows the electrical gradient formed by Na^+ movement (Marshall, 2002).

Cold temperatures can challenge the ionoregulatory machinery of fishes (Karnaky et al. 1976; Gibbons et al. 2016). This occurs because of a mismatch between the effects of low temperatures on passive ion leak and the effects on the activity of Na^+-K^+-ATPase and H^+-ATPase that are crucial for regulating ion levels (Gonzalez & McDonald, 2000). Essentially, there is a difference between the Q_{10} (the temperature coefficient, a measure of thermal sensitivity of a rate) of passive ion leak and that of the activities of the enzymes that power ionoregulation. For example, Na^+-K^+-ATPase and H^+-ATPase have a relatively high Q_{10} of 2-3, which means as the temperature drops the activity of the enzyme slows two- to three-fold for every 10^{o}C decrease (Gonzalez & McDonald, 2000). Ion leak however, has a lower Q_{10} of around 1, which indicates near thermal independence—ion leak rates do not decrease appreciably as temperature drops (Gonzalez & McDonald, 2000). Note that the Q_{10} reflects an exponential function, so the difference between Q_{10}s of 1 and 3 is substantial. Thus, in the absence of compensatory adjustments, the large decrease in ion pumping activity coincident with unchanged ion leak in the cold can result in a constant net loss (in freshwater) or gain (in seawater) of ions, leading to disturbed body ion levels and osmolarity. In fact, this compromised

ability to regulate ions is a major cause of death in the cold in fishes and is referred to as osmoregulatory dysfunction (Hurst, 2007).

It has been proposed that the challenge of ionoregulating in the cold during winter could act as a barrier to saltwater species moving in and colonizing freshwater environment at temperate and high latitudes (Lee & Bell, 1999). Freshwater at temperate latitudes often reaches lower temperatures during winter than saltwater and it is generally thought that ionoregulation in freshwater is particularly challenging for fishes because there are so few ions present in the water relative to their body compared to saltwater fish (Lee & Bell, 1999). Some of the first work done on ionoregulation in the cold was by Umminger (1970; 1971) who worked on Mummichogs (*Fundulus heteroclitus*) because they are euryhaline, able to live in both sea and freshwater. Euryhaline fishes provide great models since we are able to study them in two different salinity environments along with comparing them to species that live in one or the other. Umminger (1970) found that ionoregulation in freshwater adapted Mummichogs in near freezing temperatures was impaired with concentration of Na^+ and Cl^- being reduced. More recent studies on the impacts of winter cold on ionoregulatory capacity have been done on the euryhaline ecotypes of Three-spine stickleback (*Gasterosteus aculeatus*) (Gibbons et al. 2016). They found different growth strategies that had differing ionoregulatory strategies associated with them between freshwater and seawater populations caused by winter conditions in freshwater (Gibbons et al. 2016). One such difference between the two was the higher requirement of calcium in the seawater populations and their

lower levels of epithelial calcium channels when compared to the freshwater populations (Gibbons et al. 2016). These differences in ionoregulatory strategies allowed the freshwater ecotypes to dedicate more energy to growth over winter and thus have higher reproduction (Gibbons et al. 2016). This lead to their conclusion that winter conditions and freshwater imposed a barrier to freshwater residency (Gibbons et al. 2016). Thus, there is some evidence that certain species of fish are able to survive in freshwater but are not able to compete and establish themselves due to the challenges of ionoregulation in the cold.

I addressed the question of whether ionoregulation in winter is a challenge to freshwater colonization by comparing the ionoregulatory responses to simulated winter cold in freshwater in the Banded killifish (*Fundulus diaphanus*) and the Mummichog. These species were selected because of their close relationship, similar size, overlapping geographic range, and similar temperature tolerance (Pierce & Crawford, 1997; Fritz, 1973). Although both species are euryhaline, Banded killifish are mainly found in freshwater and Mummichogs are mainly found in saltwater – the preferred salinity habitat of the two species differs markedly (Fritz & Garside, 1974). It is also important to note that Mummichogs overwinter in saltwater as opposed to freshwater (Raposa, 2003).

Recent research on Mummichogs in seawater by Barnes et al. (2014) has found that there is a steep decline in their ability to transport ions in and out of the body as water cools below 8°C. At this point Na^+-K^+-ATPase has a decrease in their activity which may make it harder for them to properly maintain

their osmolality as is seen is Umminger's work (Barnes et al. 2014; Umminger, 1970). Due to these difficulties in the marine environment it could prove an even larger challenge for the primarily marine mummichog to ionoregulate in winter freshwater compared with the more freshwater-adapted banded killifish. Few studies besides Umminger have been done comparing ionoregulatory responses in the cold in freshwater acclimated Mummichogs, let alone in Banded killifish. Based on the hypothesis that the impairment of ion regulation during winter temperatures acts as a barrier for saltwater species to colonize freshwater habitats, I predicted that the Banded killifish would maintain a more stable level of plasma Cl^- (a proxy for ionoregulatory status) compared with the Mummichogs during exposure for up to 4 weeks to winter cold in freshwater, and that this will be associated with enhanced upregulation of Na^+-K^+-ATPase and H^+-ATPase in gill. By taking samples at day 0, 4, 15, and 28 of cold acclimation, I was able to also examine the temporal nature of the acclimation response in each species, which is not done often (Somero, 2015).

Materials and Methods

Animals

Mummichogs (0.796 ± 0.043 g mean+/-s.e.m n=45) were collected in October, 2017, using minnow traps from Sam Orr Pond, Bocabec, New Brunswick and Banded killifish (0.622 ± 0.026 g mean+/-s.e.m n=39) were collected with seine nets from the Saint John River, New Brunswick, in November, 2017. After collection, fish were transported back to the lab and held in separate tanks with flow-through freshwater (~16^{o}C). Fishes were fed daily to

satiation with trout feed, size 1.2mm (Gemma, Skretting). The fish were given a minimum of 4 weeks to acclimate to the freshwater under a winter photoperiod (9L:15D), then were transferred to the experimental tanks.

Experimental Set up

Four 38-gallon tanks per species were setup and filled with freshwater (dechlorinated City of Saint John tap water). Water composition was $[Na^+]$ = 0.2, $[Cl^-]$ 0.3, $[K^+]$ = 0.006, $[Ca^+]$ = 0.096 (mM), with hardness (as $CaCO_3$) = 5 mg/L, total dissolved carbon = 0.8 mg/L, and pH 6.8). Three of the tanks received recirculating freshwater from sumps, where the water was filtered, aerated, and cooled to 14°C using inline water chillers. These three tanks comprised the cold exposure treatment group, where temperatures were initially held at 14°C and then would be sequentially lowered over time. The fourth tanks were designated as a 'exposure control' tank and was fed with flow-through freshwater and maintained at 14°C using aquarium heaters. The purpose of the "exposure control" tank was to have a sampling group of fish under normal warm conditions throughout the multi-week cold exposure, so as to account for potential temporal effects on measured variables. 20 Mummichogs were transferred to each of the three experimental tanks with the same being done for Banded killifish. The Mummichog and Banded killifish exposure control tanks both had 12 individuals transferred into them. The fish were then given 2 weeks to acclimate to the experimental set up at 14°C under a winter photoperiod (9L:15D).

Cold temperature exposure and sampling

Sampling (see below) of control fish at 14°C was conducted at the start of the experiment from the experimental tanks. The temperature in the experimental tanks was then dropped by 1°C per day from 14°C to 4°C and then held at 4°C for four weeks. Samples were taken 24 hours after 4°C was reached (cold exposure day 0), as well as at 4 days, 14 days, and 28 days of holding at 4°C. Individuals were randomly sampled from the three exposure tanks. Fish from the control tank were sampled at the end of the experiment. For sampling, the fish were anaesthetized with MS-222 (0.2 g/l + 0.2 g/l $NaHCO_3$) and weighed. A blood sample was taken by cutting the tail and using a heparinized hematocrit tube to collect the mixed arterial-venous blood. Blood was centrifuged in a hematocrit centrifuge to measure hematocrit (packed red blood cell %) and isolate the plasma. The plasma was frozen at -20°C and then transferred to a -80°C freezer until analyzed. The liver was weighed to calculate the hepatosomatic index (HSI), liver to body weight ratio, and was then frozen in liquid nitrogen. The gill basket, intestine, and a white muscle transverse section (taken behind the second dorsal fin) were also quickly dissected and frozen in liquid N_2. All samples were stored at -80°C until analysis.

Measurement of plasma Cl^-

Plasma Cl^- levels were measured using the colorimetric mercuric thiocyanate method (Zall et al., 1956). The level of Cl^- in plasma was used as a

proxy for ionoregulatory status because under normal conditions teleost fishes tightly regulate their internal Cl^- concentrations.

Measurement of gill enzyme activities

Whole gill baskets were manually homogenized in 20 volumes of ice-cold SEID buffer (150mM sucrose, 10mM EDTA, 50mM imidazole, 0.1% sodium deoxycholate, pH 7.3) using a ground glass Tenbroeck homogenizer. The homogenate was transferred into microfuge tubes and centrifuged for 1 minute at 5000 g. The supernatant was removed and frozen at -80^{o}C until enzyme measurement (within 1 week).

The maximal activities of Na^+-K^+-ATPase and H^+-ATPase in the gill were measured following a method based on Hawkings et al. (2004) and Kültz and Somero (1995). The assay medium contained 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 50 mM imidazole, 3 mM ATP, 2 mM PEP, 0.21 mM NADH, and excess PK and LDH (pH 7.5). Three reactions were measured in triplicate per sample: (1) control reaction of total ATPase activity using gill supernatant + the above assay medium; (2) gill supernatant + assay medium + ouabain (0.5 mM), a selective blocker of Na^+-K^+-ATPase; and (3) gill supernatant + assay medium + ouabain (0.5 mM) + N-ethylmaleimide (1 mM), a selective blocker of H^+-ATPase. The reaction was observed by following the decrease in absorbance at 340nm on a Molecular Devices SpectraMax 190 at 25°C. Na^+-K^+-ATPase activity was calculated as the difference in rates between reactions 1 and 2 (i.e. the fraction of total ATPase activity blocked by ouabain), and H^+-ATPase activity was calculated as the difference in rates between reactions 2 and 3 (i.e. the

fraction of ouabain-insensitive ATPase activity blocked by N-ethylmaleimide). Ouabain is included in the assay for H^+-ATPase activity in order to eliminate Na^+-K^+-ATPase activity, thus providing a cleaner baseline to detect H^+-ATPase activity. Protein in the gill supernatant was quantified using the Bradford assay (Sigma-Aldrich) and all enzyme activities are expressed as µmol ADP/hour/mg protein. All reagents were purchased from Sigma-Aldrich or Fisher Scientific.

Statistics

The values for Cl^- and enzyme activities for the 14^{o}C control fish sampled at the start of the experiment and those sampled at the end of the experiment were compared with a Student's t-test; there was no significance difference between the start and end control groups, so the two groups were pooled into one 14^{o}C control group. A two-way ANOVA with post hoc tukey test was run to determine the effect of species and time of exposure at 4°C on the body weight, hepatosomatic index**,** hematocrit, plasma chloride levels and the activity of Na^+-K^+-ATPase and H^+-ATPase. All statistics were run using RStudio. A few individual fish did not have a complete set of chloride, Na^+-K^+-ATPase, or H^+-ATPase measurements and were removed from the analysis. Significance was accepted at $p<0.05$.

Results

Mortality was low. Only four mortalities occurred, all of which were Mummichogs, with one dying at 14°C on the fifth day after being transferred to the experiment tanks before cooling had begun, one dying five days into

cooling, and two dying after 20 days and 24 days at 4^{o}C. When comparing the body weight between the two species I found that Mummichogs were significantly heavier than the Banded killifish ($p<0.01$) (Table. 1). The body weights did not significantly change over the course of the experiment. The Mummichogs hematocrit and HSI was significantly higher then the Banded killifish ($p< 0.05$) but no difference was found between the Mummichog and Banded killifish control (Table. 1). The hematocrit and HSI did not change over the course of the experiment in either species.

No difference was found in plasma chloride levels between the species or over time at 4^{o}C (Fig. 1). Na^{+}-K^{+}-ATPase enzyme activity and H^{+}-ATPase were not different between species and did not change significantly during the cold acclimation (Fig. 2, Fig. 3).

Table: 1: Body weight, Hepatosomatic index (HSI), and Hematocrit of freshwater-acclimated Banded killifish (*Fundulus diaphanus*) and Mummichogs (*Fundulus heteroclitus*) at the control temperature of 14°C and after 0, 4, 14, and 28 days of acclimation to 4°C. Data are means ±SE.

	Body Weight (g)		**HSI (%)**		**Hematocrit (%)**		**Sample size (n)**	
	Banded killifish	Mummichog	Banded killifish	Mummichog	Banded killifish	Mummichog	Banded killifish	Mummichog
Control (at 14°C)	0.598 ±0.021	0.881 ±0.074	3.61 ±0.26	4.30 ±0.38	32.9 ±1.1	35.2 ±1.2	12*	15**
0 (at 4°C)	0.618 ±0.057	0.887 ±0.140	4.69 ±0.29	5.64 ±0.36	32.1 ±1.4	38.1 ±2.1	7	9
4 (at 4°C)	0.721 ±0.037	0.796 ±0.048	3.32 ±0.56	5.50 ±0.46	31.0 ±2.0	40.3 ±4.6	8	8
14 (at 4°C)	0.700 ±0.170	0.744 ±0.049	3.13 ±0.53	4.53 ±0.87	37.1 ±6.5	40.1 ±3.0	5***	4
28 (at 4°C)	0.555 ±0.059	0.618 ±0.039	2.92 ±0.33	4.98 ±0.34	29.1 ±1.3	35.6 ±2.6	7	10

*n=11 for Hematocrit **n=14 for Body Weight, HSI, and Hematocrit ***n=4 for Hematocrit

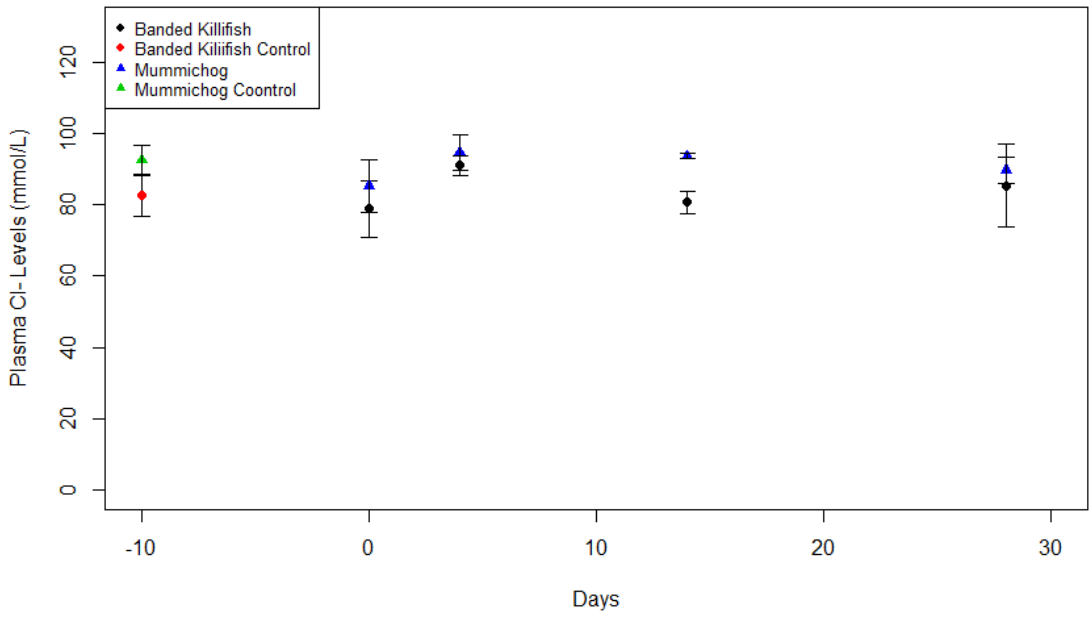

Figure 1: Plasma chloride levels in freshwater-acclimated Banded killifish (*Fundulus diaphanus*) and Mummichogs (*Fundulus heteroclitus*) at the control temperature of 14°C and after 0, 4, 14, and 28 days of acclimation to 4°C. For sample size refer to Table 1.

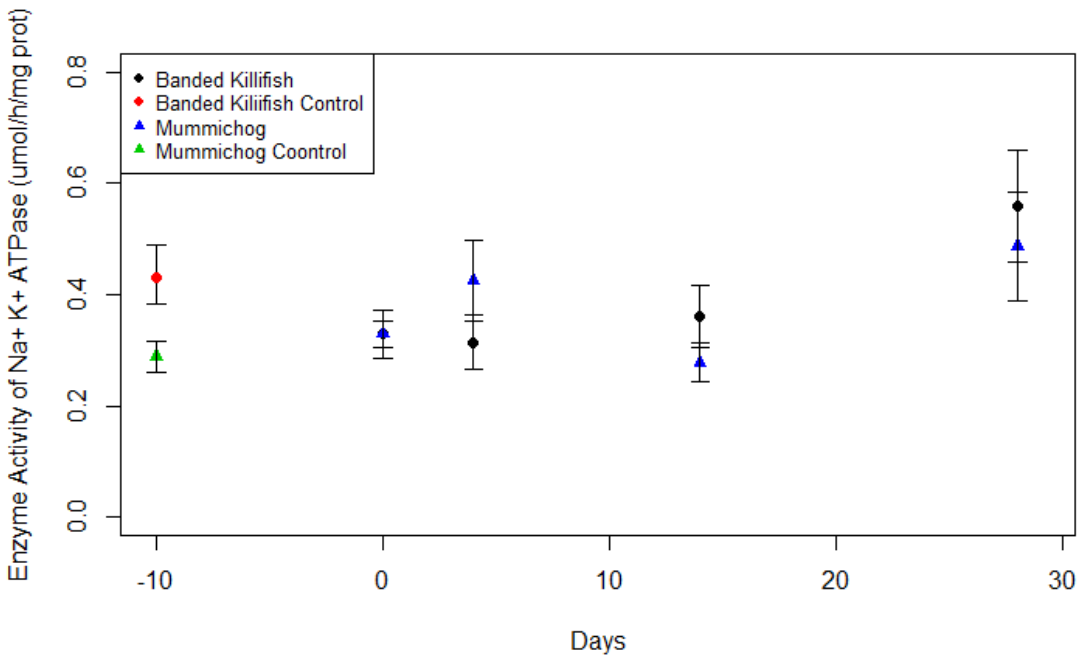

Figure 2: Maximal enzyme activities of Na^+-K^+-ATPase in gills of freshwater-acclimated Banded killifish (*Fundulus diaphanus*) and Mummichogs (*Fundulus heteroclitus*) at the control temperature of 14°C and after 0, 4, 14, and 28 days of acclimation to 4°C. Data are means ±SE. For sample sizes refer to Table 1.

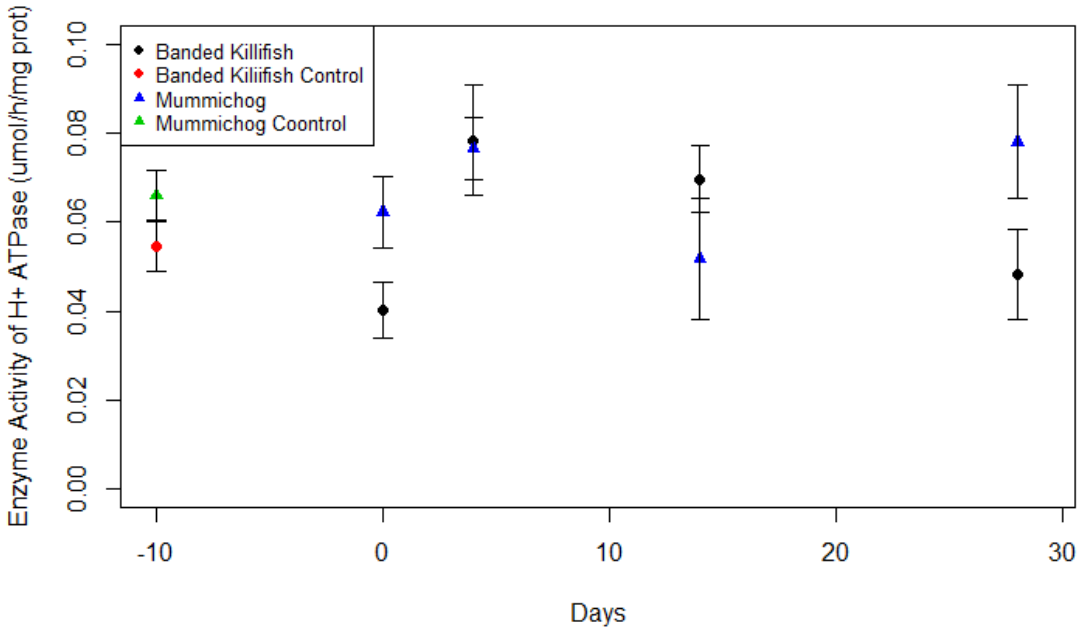

Figure 3: Maximal enzyme activities of H^+ ATPase in gills of freshwater-acclimated Banded killifish (*Fundulus diaphanus*) and Mummichogs (*Fundulus heteroclitus*) at the control temperature of 14°C and after 0, 4, 14, and 28 days of acclimation to 4°C. Data are means ±SE. For sample sizes refer to Table 1.

Discussion

Using a comparison of the responses to winter cold in freshwater in two closely related killifish that differ in their salinity preference, I tested the hypothesis that the challenge of ionoregulation in cold freshwater acts as a barrier to freshwater colonization by fishes. Our results did not support the hypothesis. Plasma chloride was similar between Banded killifish and Mummichogs and was not affected in either species by up to 28 days of acclimation to 4^{o}C (Fig. 1), indicating that osmotic status was not perturbed by winter cold. Furthermore, I observed no effects of cold acclimation on activities of the key ionoregulatory enzymes Na^{+}-K^{+}-ATPase and H^{+}-ATPase in gill, which were similar between species. Thus, there is no evidence that winter cold results in perturbation of ionoregulation or compensatory regulatory responses in either species, even in the primarily marine mummichog.

Plasma chloride levels were similar to those found in Mummichogs and Three-spine sticklebacks in cold freshwater (Umminger 1971; Gibbons et al. 2016). I expected that chloride levels would be lower in the cold, at least initially when sampled at 4°C on day 0, but this was not found which differs from Umminger's (1971) findings for mummichogs, where decreases in chloride were observed after cold acclimation at 4oC or near freezing temperatures for up to 16-18 weeks. This discrepancy may be a result of the 4-week acclimation time in my experiment being shorter than the acclimation time used by Umminger (1971), so it is possible that cold-induced difficulties in ionoregulation in freshwater-acclimated mummichogs only occur after longer durations of

exposure to cold. Nonetheless, my exposure time of 28 days is a typical thermal acclimation period to reach a new steady-state (Sidell et al. 1973) and saw no evidence whatsoever of any trend towards perturbed ionoregulation. Another possible reason for this discrepancy could be that Umminger's fish may have been a hybrid of the northern and southern subspecies of Mummichogs. The range of the two subspecies meet between Connecticut and New Jersey with hybrids of the two being found in this area (McKenzie et al. 2015). This is important because the southern subspecies have been observed to have difficulties reducing Cl^- loss when moved to freshwater while the northern subspecies was able to minimize chloride loss to the environment (Scott et al. 2004). Hybridization of the subspecies could lead to offspring being less well adapted to surviving in freshwater. Thus, while I used the northern subspecies in my experiment, Umminger (1971) may have been studying a hybrid which might explain why he observed a significant perturbation in plasma Cl- whereas I did not. Overall, our results suggest that although northern Mummichogs prefer marine or estuarine habitats, they are able to control their ionoregulation as effectively as the Banded Killifish at low temperatures characteristic of winter.

I predicted that in order to maintain stable chloride levels in the cold the Mummichogs and Banded killifish would have to upregulate activities of enzymes related to inonoregulation. This would be required given the loss of effectiveness at transporting ions in colder temperatures as seen by Barnes in seawater (Barnes et al. 2014). The gill Na^+-K^+-ATPase activities I measured are similar to those previously measured in Mummichogs in freshwater (Scott et al.

2004), and the gill H^+-ATPase activities are similar to levels measured previously in freshwater fishes (Kültz & Somero, 1995; Hawkings et al., 2004). Contrary to our prediction, I found that Na^+-K^+-ATPase and H^+-ATPase activities were unchanged during the 28-day acclimation to 4°C and were also similar between species (Fig. 2 & 3). Thus, there is no evidence of cold-induced regulatory response in gill to maintain stable ion levels. Past studies however have seen an increase in activity after 28 days exposure to 1oC in freshwater in goldfish (*Carassius auratus*) (Paxton & Umminger, 1983). Similarly, increases in Na^+-K^+-ATPase have been observed in Atlantic cod (*Gadus morhua*) and Rainbow trout (*Oncorhynchus mykiss*) when exposed in saltwater for 17 days at 1°C and 21 days at 2°C, respectively (Staurnes et al. 1994; McCarty & Houston 1977). To my knowledge, my measurements are the first done on the responses of gill H^+-ATPase activity to cold in fish. Thus, Mummichogs and Banded killifish may be using a different method to control the leak of ions out in cold freshwater.

Gill remodeling, which I did not investigate, may help explain the maintenance of ion levels without changes in regulatory enzymes. At cold temperatures in saltwater Mummichogs will undergo gill remodeling to reduce epithelial surface area which will decrease passive ion leak in (Barnes et al. 2014). This is done by filling the space between the lamellae with epithelial cells (Barnes et al. 2014). These changes in the gill can occur quickly with the northern subspecies of Mummichogs, which I used, being able to stop any loss of ions that can not be replaced in 24 hours after transfer into freshwater (Scott

et al. 2004). This quick remodeling of the gill could also help with ion loss in the cold freshwater which would not result in enzyme activity being needed to increase. Studies on gill remodeling in cold freshwater have not been conducted on Mummichogs or Banded Killifish though, and this should be researched in the future. A second factor that was not measured here is the activity of Na^+-K^+-ATPase and H^+-ATPase found in the kidneys. An increase in ion reabsorption by the kidneys in the cold could help to maintain ionoregulation since it is a site of significant ion loss in freshwater (Wood & Marshall, 1994).

Fish health and stress was probably not a factor that effected the results. Mortalities were low with only the four-reported, 3.2% of fish sampled, spread out over the experiment. Only Mummichog mortalities were observed but the low death count does not allow us to make an assumption of it being due to exposure to the cold freshwater. No change in body weight, hematocrit, or HSI was seen in either species over the acclimation period, suggesting that the condition of the fish was relatively unaffected by cold acclimation (Table 1). Reduced feeding was observed as temperatures cooled but this was expected as metabolic rate slows at cold temperature (Schulte et al. 2011).

In summary, I did not find support for the hypothesis that cold freshwater acts as a barrier to freshwater colonization by impairing ionoregulation in fishes. I did not find any evidence that plasma chloride (an indicator of osmotic status) was more perturbed in the mummichog compared with the banded killifish, or that energetically expensive compensatory increases in ionoregulatory enzyme activities occurred in either species. Thus, in cold freshwater, the euryhaline

Mummichog, which primarily lives in saltwater and overwinters in saltwater, was able to control ion levels equally as well as the Banded Killifish, which primarily lives in freshwater and overwinters in freshwater. My conclusion is consistent with that of Gibbons et al. (2016), who found that plasma chloride levels and Na^+-K^+-ATPase gene expression did not show clear differences between marine and freshwater ecotypes of Three-spine stickleback during winter cold in freshwater. Physiological constraints on growth in the cold, and a potentially linked inability to properly regulate calcium uptake from the environment during winter cold, may be a more important constraint on freshwater invasion in fish (Gibbons et al., 2016). Future studies should examine calcium regulation in cold in mummichogs and banded killifish. Also, studies are needed to investigate if gill remodeling occurs differently between the two killifish species in the cold as well as whether there are species-specific differences in the function of the kidneys in ionoregulation. In general, I found a lack of data on ionoregulation in cold freshwater and future studies should be conducted to fill this knowledge gap.

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