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Osmotic versus adrenergic control of ion transport by ionocytes of *Fundulus heteroclitus* in the cold

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ABSTRACT

In eurythermic vertebrates, acclimation to the cold may produce changes in physiological control systems. We hypothesize that relatively direct osmosensitive control will operate better than adrenergic receptor mediated control of ion transport in cold vs. warm conditions. Fish were acclimated to full strength seawater (SW) at 21 °C and 5 °C for four weeks, gill samples and blood were taken and opercular epithelia mounted in Ussing style chambers. Short-circuit current (I_{sc}) at 21 °C and 5 °C (measured at acclimation temperature), was significantly inhibited by the α_2-adrenergic agonist clonidine but the ED_{50} dose was significantly higher in cold conditions (93.8 ± 16.4 nM) than in warm epithelia (47.8 ± 8.1 nM) and the maximum inhibition was significantly lower in cold (−66.1 ± 2.2%) vs. warm conditions (−85.6 ± 1.3%), indicating lower sensitivity in the cold. β-Adrenergic responses were unchanged. Hypotonic inhibition of I_{sc}, was higher in warm acclimated (−95%), compared to cold acclimated fish (−75%), while hypertonic stimulations were the same, indicating equal responsiveness to hyperosmotic stimuli. Plasma osmolality was significantly elevated in cold acclimated fish and, by TEM, gill ionocytes from cold acclimated fish had significantly shorter mitochondria. These data are consistent with a shift in these eurythermic animals from complex adrenergic control to relatively simple biomechanical osmotic control of ion secretion in the cold.

1. Introduction

Fundulus heteroclitus, (mummichog) are euryhaline and eurythermic teleost fish able to acclimate to extreme salinity, from freshwater to over 3× seawater habitats (Griffith, 1974) and temperatures ranging from −1.5 °C to 38 °C (Fangue et al., 2009). Previously, we determined that acclimation of mummichogs to the cold, 5 °C, slows down but does not stop NaCl secretion by the mitochondrion-rich cells (ionocytes) of the opercular epithelium and gill of seawater animals (Buhariwalla et al., 2012; Barnes et al., 2014). Importantly, acclimation to the cold for >30 days causes significant changes in fatty acid saturation, specifically a reduction in C18:0 and an increase in monounsaturated n−9 fatty acids in membranes from liver (Buhariwalla et al., 2012) and gill epithelium (Barnes et al., 2014). This demonstration of phenotypic plasticity is also seen in Antarctic fish and considered a mechanism to regulate metabolic and cardiovascular function in correspondence to temperature fluctuations (Guderley, 2004). These changes also help maintain sensitivity of the osmotic control pathway that controls NaCl secretion, a pathway that involves integrin α/β mechanosensing of changes in plasma membrane stretch, transduction to a kinase cascade that includes proto oncogene c-SRC kinase (c-SRC), focal adhesion kinase (FAK), ste20-related kinase (SPAK) and oxidative response kinase (OSR1) that ultimately activates Na^+, K^+, $2Cl^-$ cotransporter (NKCC) in the basolateral membrane of ionocytes and increases NaCl secretion (Marshall et al., 2005, Marshall et al., 2008). We also determined that a similar pathway involving FAK may also activate the anion channel cystic fibrosis transmembrane conductance regulator (CFTR) in the apical membrane, thus the Cl^- transcellular secretion pathway can be rapidly activated by the simultaneous phosphorylation and activation of transporters in basolateral and apical membranes (Marshall et al., 2009). These rapid osmotically-mediated pathways have convergent points, such as 14-3-3 protein (Kültz et al., 2001) and can evoke longer term responses through transcription factors, such as OSTF1 and TFIIB (Fiol and Kültz, 2005; Kültz et al., 2013). Osmosensing control systems that modulate ion transport rates during osmotic stresses include osmosensor proteins, osmotic effector proteins and osmotically activated transcription factors that collectively have been linked with the emergent evolution and specialization of euryhalinity in teleost fish (Evans, 2010).

NaCl secretion is also under complex neuroendocrine control and is stimulated by β-adrenergic agonists, Urotensin I, vasoactive intestinal polypeptide and glucagon, all via cAMP, and is inhibited by α_2-adrenergic agonists, Urotensin II, acetylcholine, endothelin, eicosanoids and mediators that increase intracellular Ca^{2+}, such as ionomycin (reviewed by Evans, 2002, Evans, 2010, Hyndman et al., 2006, Evans et al., 2005). The opposing effects of α_2- and β-adrenergic pathways on Cl^- secretion are well-known (May and Degnan, 1985; Marshall

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et al., 1993) and both pathways involve multiple protein interactions and intracellular second messengers (Ca^{2+} and cAMP, respectively). Typically, β-adrenergic activation involves hormone-receptor binding at the plasma membrane, transactivation of adenylate cyclase, production of the second messenger cAMP from ATP, diffusion of cAMP intracellularly, activation of protein kinase A (PKA) and ultimately phosphorylation and activation of NKCC and CFTR in the basolateral and apical membranes, respectively. In the opposite response, the cholinergic and adrenergic inhibitory pathways appear to converge at the level of the transporters (May and Degnan, 1985). Whereas α- and β-adrenoceptors are present in an isolated nerve-opercular-epithelium preparation, the dominant response to nerve stimulation is activation of α_2-adrenergic receptors and rapid inhibition of I_{sc} (Marshall, 2003; May and Degnan, 1985). This response we interpret as physiologically important in context of the animals moving from seawater to more dilute environments and needing to rapidly decrease Cl^- secretion (Marshall, 2003). The α_2-adrenergic activation involves neurotransmitter receptor binding, transactivation of phospholipase C, production of inositol tris-phosphate (IP3) and increase in cytosolic Ca^{2+}, a complex cascade that, in the ionocytes culminates in dephosphorylation and deactivation of NKCC at the basolateral and CFTR at the apical membrane (Lurman et al., 2012). The hormone-receptor kinetics and large number of amplification steps in the adrenergic pathways would make these pathways subject to cold-induced attenuation or failure, unless there were extensive thermoacclimation at several levels of the cascades.

Adrenergic responses to cold acclimation vary by tissue and fish species. Rainbow trout heart becomes more sensitive to β-adrenergic stimulation of heart rate and contractility in the cold (Aho and Vornanen, 2001; Keen et al., 1993). Similarly, hepatocytes from rainbow trout have modulation of receptor number and a slower but more sensitive response to adrenaline in the cold (McKinley and Hazel, 1993, 2000). In contrast, arterioles from cold acclimated (1 and 5 °C) rainbow trout become less sensitive to β-adrenergically mediated vasodilation, compared to 10 °C acclimated controls (Costa et al., 2015). Meanwhile, in cod heart, cold acclimation (0 and 4 °C) was not different from warmer (10 °C) inotropic responses to norepinephrine (Lurman et al., 2012). Finally, Antarctic notothenid fishes seem to have evolved almost pure cholinergic control of heart and spleen, with no responsiveness to catecholamines (Davison et al., 1997). With examination of other fish and more diverse systems, perhaps a pattern of cold acclimation and adrenergic responsiveness will emerge.

Here, mummichogs were acclimated to warm (20 °C) or cold (5 °C) seawater, mimicking summer and winter conditions in the estuary, then tested in vitro for the up- and down-regulation of Cl^- secretion by adrenergic and osmosensitive pathways at these temperatures. We hypothesized that cold acclimation would interfere with the adrenergic mediated up- and down-regulation of Cl^- secretion because of the complexity of the neurohormonal mediated second messenger pathways, whereas the relatively direct osmotic regulation of Cl^- secretion would be more effective in the cold, assuming that the animals were acclimated to these conditions and maintained membrane fluidity. In addition, gill tissue was taken to examine ionocyte ultrastructure, as previous studies on cold acclimation described changes in mitochondrial structure and density in cold FW acclimated fish and our study provided an opportunity to examine the same for SW fish (St-Pierre et al., 1998; Mitrovic and Perry, 2009; Egginton et al., 2000).

The present study tests whether neural regulatory responses of active Cl^- secretion by ionocytes are still as sensitive and responsive in the cold, compared to the relatively simple osmotic responses. We found that epithelia from cold acclimated animals have impaired responsiveness to adrenergically-mediated inhibition, whereas the tissues remain fully responsive to osmotic stimulated regulation of Cl^- secretion, underscoring the importance of cell volume osmotic regulation of ion transport in the cold.

2. Materials and methods

2.1. Research animals

Male and female Northern killifish or mummichog (*Fundulus heteroclitus*) were collected in June and July 2015 from Ogdens Pond in Antigonish County, Nova Scotia and transferred to the St. Francis Xavier Animal Care Facility and were kept in 450 L aquaria in natural seawater (30 ppt) under a 12L:12D hour photoperiod. Warm acclimated fish were kept at ambient temperature (20–22 °C). Prospective cold acclimated fish were initially kept at ambient temperature; the temperature was then decreased by 2 °C, every 2 days, until the final acclimation temperature of 5–6 °C was reached and maintained for a minimum of 30 days. All fish were fed Nutrafin fish food flakes (R. C. Hagen, Montreal, Quebec, Canada) every morning, and alternating live chopped mealworms, freeze dried *Tubifex* worms and freeze dried brine shrimp in the afternoon. The aquarium water was changed by 1/3 tank volume weekly.

The fish were euthanized either by pithing, or by anaesthetization (MS-222 in 0.9% NaCl 2 $g \cdot L^{-1}$, adjusted to pH 7.0) followed by exsanguination. All experimental procedures were approved by the St. Francis Xavier Animal Care Committee (protocol 15-001-N) and followed Canadian Council on Animal Care guidelines.

2.2. Bathing solutions

Cortland saline, composed of (all in mmol L^{-1}): 159.9 NaCl, 2.55 KCl, 1.56 $CaCl_2$, 0.93 $MgSO_4$, 17.85 $NaHCO_3$, 2.97 NaH_2PO_4, and 5.5 glucose (all chemicals from Sigma-Aldrich, St Louis, Mo, USA), was used as the isotonic bathing solution in all electrophysiology experiments. All bathing solutions were bubbled with a 99% O_2/1.0% CO_2 gas mixture before use (pH 7.6–7.8).

Hypotonic Cortland's was made by diluting regular Cortland's with high quality (HQ) water (18 MΩ ion exchange water, Barnstead E-pure®). Mixtures of 200 mL total volume were mixed to decreasing total osmolalities of 354 $mOsm \cdot kg^{-1}$ Cortland:HQ water 194 mL:6 mL, 330 $mOsm \cdot kg^{-1}$ 182:18, 315 $mOsm \cdot kg^{-1}$ 172:28, 279 $mOsm \cdot kg^{-1}$ 154:46, 258 $mOsm \cdot kg^{-1}$ 142:38, and 238 $mOsm \cdot kg^{-1}$ 134:66.

Hypertonic Cortland's was made by adding mannitol (Sigma-Aldrich), an osmolyte that is not metabolized, to full strength Cortland's. Stock solutions were made to a final concentration in 200 mL at osmolalities of 365 $mOsm \cdot kg^{-1}$ (0.364 g mannitol), 385 $mOsm \cdot kg^{-1}$ (1.093 g mannitol), 415 $mOsm \cdot kg^{-1}$ (2.186 g mannitol) and 445 $mOsm \cdot kg^{-1}$ (3.280 g mannitol). Osmolality was measured by vapor pressure osmometry (Vapro® vapor pressure osmometer (Wescor, Inc., Logan, Utah). Both the hypotonic and hypertonic bathing solutions represent a physiological range of osmolality encountered in nature by mummichogs (Griffith, 1974).

2.3. Electrophysiology

Ussing chambers were used to measure ion transport in both warm and cold acclimated fish. Ussing chambers consist of two hemi chambers, which surround either side of the opercular epithelia. Under a dissecting microscope, fine forceps (Dumont no. 5) were used to dissect the opercular epithelia from the opercular bone of the fish. Tissues from cold-acclimated fish were dissected on icepack and the temperature of all solutions was kept at 5.0 °C throughout the experiment. The epithelia were placed between two Plexiglas® inserts with a circular aperture with an area of 0.125 cm^2. The inserts were covered in silicone high vacuum grease to create an electrical seal with the chambers. Once the inserts were securely fastened inside the chamber, both hemi chambers were simultaneously filled with isotonic Cortland's saline. Surrounding the hemi chambers were water jackets, which had water mixed with antifreeze circulated through them by a bath circulator (Polyscience, Niles, IL, USA) to control temperature. Temperature

was maintained at 22 °C for the opercular epithelia of warm acclimated fish and 5 °C for that of cold acclimated fish, and measured by a thermometer in the hemi-chamber on the mucosal side of the epithelia. Oxygen flow to both sides of the epithelia was maintained by a stirring magnet in each hemi-chamber.

The hemi chambers were attached to a current voltage clamp (World Precision instruments, D. Lee Co., Sunnyvale, Ca), which connected two circuits, a current passing circuit, and a voltage measuring circuit. Pulses of current (10 μA, 1.0 s) were sent through the epithelia by the current passing circuit once every minute, creating voltage deflections. Transepithelial potential difference (V_t, mV), and the voltage deflections (mV) were measured by the voltage measuring circuit using two Ag/AgCl half cells connected to the chamber. These measurements were used to calculate membrane resistance ($\Omega \cdot cm^2$) and short-circuit current (I_{sc}, $\mu A/cm^2$), equal to active ion secretion across the epithelia. Output was recorded (iWorx systems Inc., modelIX-TA-220, Dover, NH, USA) and signals collected using data acquisition software (LabScribe3, iWorkx).

2.4. Drug additions and solution exchanges

Isoproterenol, a β-adrenergic agonist, and clonidine an α_2-adrenergic agonist (both from Sigma-Aldrich), were prepared fresh daily as 1.0 and 0.1 $mg \cdot mL^{-1}$ stock solutions in 0.9% NaCl. All drugs were added to the hemi chamber on the serosal side.

Once a new epithelium was mounted to the Ussing chambers and bathed in full strength Cortland's saline, it was allowed to reach a steady state (20–30 min). To exchange bathing solutions, 30 mL of new solution was added to the bottom of the 3.3 mL hemi chamber, while the old solution was removed from the top by suction. All drug additions and solution exchanges were performed at time intervals of approximately 7 min, sufficient to reach a new steady-state, for the epithelia of warm acclimated fish and 21 min for cold acclimated fish, to allow for potentially slower responses in the cold.

Two treatment groups were used to measure ion transport regulation by the osmotic and adrenergic pathways. Unstimulated opercular epithelia have high levels of ion transport, thus inhibition was performed first, followed by stimulation. In each treatment, ion transport was inhibited by one pathway, and then stimulated by the other, presumably independent pathway. In the first treatment group, solution exchanges were performed with a dilution series (in $mOsm \cdot kg^{-1}$) starting with full strength Cortland 355 $mOsm \cdot kg^{-1}$ to 354, 330, 315, 279, 258 and 238 $mOsm \cdot kg^{-1}$. Under hypotonic conditions (238 $mOsm \cdot kg^{-1}$), serial additions of β-adrenergic agonist (isoproterenol) were then performed at concentrations of 1, 3.2, 10, 32, 100 and 320 nM, (the maximally effective dose). Dose response curves were generated based on the changes in I_{sc} generated by these additions.

In the second treatment group, serial additions of α_2-adrenergic agonist (clonidine) were performed at concentrations of 1, 3.2, 10, 32, 100 and 320 nM (the dose at which transport was typically inhibited completely). Dose response curves were also generated for clonidine. Solution exchanges were then performed with increasingly hyperosmotic Cortland's (in $mOsm \cdot kg^{-1}$, measured by vapor pressure osmometry), from regular Cortland's 355 $mOsm \cdot kg^{-1}$, to 385, 415, and finally 445 $mOsm \cdot kg^{-1}$.

2.5. Plasma osmolality and cortisol

After anaesthetization, blood samples were collected from the caudal vessels into heparinized capillary tubes. The samples were then centrifuged and the plasma was separated into microcentrifuge tubes and frozen immediately at −20 °C. After approximately 90 days, the samples were defrosted, mixed and centrifuged at 1500 × *g* for 3 min. An enzyme-linked immunosorbent assay in 96 well plate (Diametra, Eagle Biosciences, Inc. Nashua, NH) was performed in duplicate on the plasma samples to measure blood cortisol following manufacturer's instructions with the exception that plasma volume was halved (10 μL). Colored product was read spectrophotometrically (Wallac Victor2, model 1420 plate reader). Blood osmolality was also measured in duplicate in a Vapro® vapor pressure osmometer (Wescor, Inc., Logan, Utah).

2.6. Electron microscopy

The second gill bar from the left side of each mummichog (n = 3 animals for both warm- and cold-acclimated treatments), was dissected and immersed in ice-cold fixative (2% glutaraldehyde, 2% formaldehyde in 0.1 M phosphate buffer pH 7.2 (PB)) and stored at 4 °C for 5–96 h. The tissue was then rinsed in PB and the gill filaments cut away from the gill bar. The filaments were immersed in 1% osmium tetroxide for 1 h, rinsed with PB or distilled water, and stored at 4 °C for 1–7 days. They were stained, en bloc, with 4% aqueous uranyl acetate for 45 min and stored at 4 °C overnight. The tissue was then dehydrated through an ascending series of ethanol and immersed in propylene oxide (2 times at 10 min per change) before being gradually infiltrated with EMBED 812 epoxy resin over three days. Individual filaments were placed in plastic molds, covered with resin and cured at 60 °C for two days.

One-micron thick sections were cut on a Porter Blum Sorval microtome, stained with 1% toluidine blue and inspected to ensure proper tissue orientation (longitudinal sections of gill filaments). Thin sections were cut, from one filament per animal, using a Reichert microtome and stained in 4% aqueous uranyl acetate (1 h) and lead citrate (4 min). Profiles of mitochondria rich cells (MRC ionocytes) were photographed on a Philips 410 TEM at 80 kV with a Kodak L 12C CCD camera using MaxIm DL 5 software at 17,000 times magnification.

The profiles of three ionocytes were taken from each of two gill filaments from each fish for a total of six samples per fish. The six profiles were coded and the mitochondrial measurements performed by an investigator (LG) unfamiliar with the codes to minimize operator bias. The resulting micrographs were viewed in Adobe Photoshop CS 5.1 where the length and width of each mitochondrial profile, from one cell profile, was measured using a ruler tool that was calibrated to the magnification factor. The results were entered into Microsoft Excel 2010 where the mean length was calculated. The number of mitochondria profiles surveyed, per cell profile, ranged from 22 to 150 with a mean of 84.

2.7. Statistical analysis

Data are expressed as mean ± one s.e.m. A two-way ANOVA followed by a multiple comparison a posteriori tests were used to analyze the changes in I_{sc} with the addition of the transport stimulators and inhibitors. Percent decrease in I_{sc} was analyzed using a Mann Whitney *U* test. Graphpad Prism™ version 7 was used for curve fitting to determine the effective dose at 50% (ED_{50}) and for statistical analysis. ED_{50} values for isoproterenol and clonidine were extracted from the individual dose response curves and compared using an unpaired two tailed *t*-test. ED_{50} for hypotonic effects were extracted by hand from individual dose response curves.

3. Results

3.1. I_{sc} changes

3.1.1. Clonidine and isoproterenol

We compared the changes in I_{sc} between the opercular epithelia of warm and cold acclimated fish (Fig. 1) in response to A) hypotonic Cortland's saline followed by B) isoproterenol and, in a separate set of opercular epithelia, C) inhibition by clonidine followed by D) hypertonic Cortland's saline. The stimulation of I_{sc} by isoproterenol was significant in both acclimation treatments but the increase in

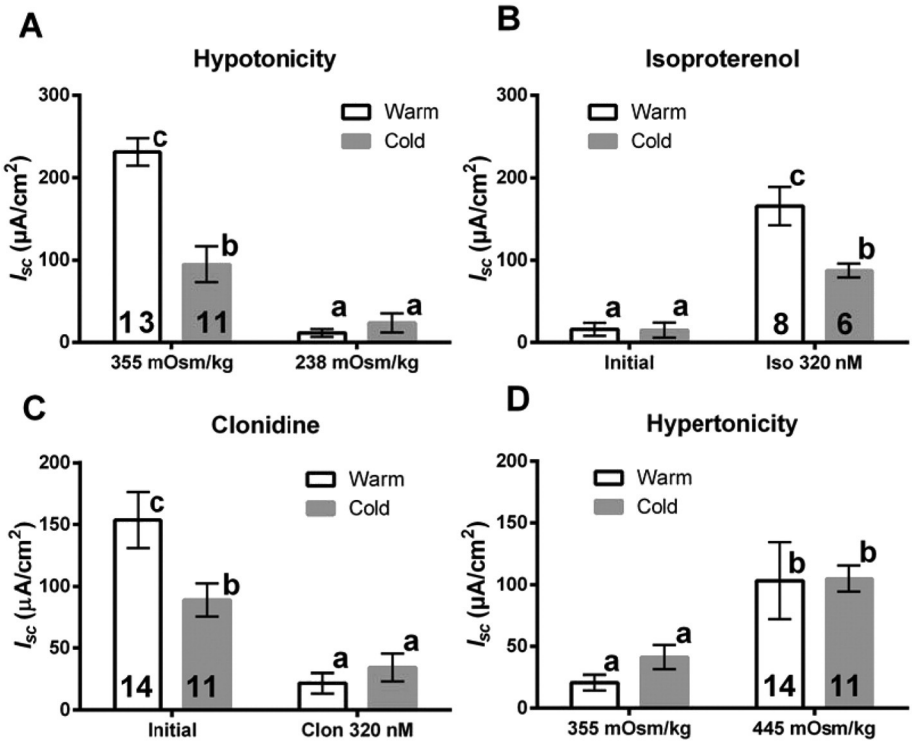

Fig. 1. Mean ± one s.e.m. I_{sc} changes in the opercular epithelia of warm (20 °C) and cold (5 °C) acclimated fish in response to A) bathing solution exchanges with increasingly hypotonic Cortland's saline followed by B) additions of isoproterenol, or C) addition of clonidine followed by D) bathing solution exchanges with increasingly hypertonic Cortland's saline. Dissimilar letters indicate significant difference ($P < 0.05$ or better) one-way ANOVA with Tukey a posteriori test.

warm acclimated fish was +10.3-fold, compared to +5.8-fold in the cold acclimated fish and the final I_{sc} reached was higher in the warm acclimated epithelia (Fig. 1B, 2-way ANOVA P values isoproterenol <0.0001, temperature <0.02, interaction <0.02). The inhibition of I_{sc} by clonidine was significant in warm and cold acclimated fish, but more profound in warm acclimated fish, with a total inhibition of −

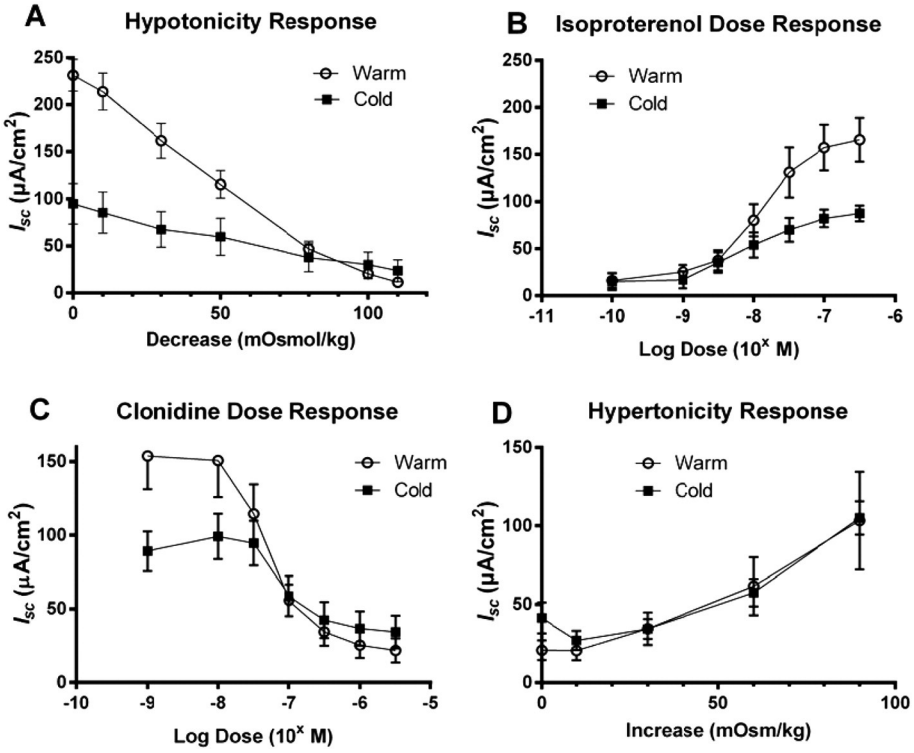

Fig. 2. Dose response I_{sc} for of the opercular epithelia from warm (20 °C) and cold (5 °C) acclimated fish exposed to A) serial dilution of the saline to hypotonic saline (n = 13 warm, 11 cold), B) serial additions of isoproterenol (n = 8 warm, 6 cold), C) serial additions of clonidine (n = 14 warm, 11 cold) and D) serial flow through changes with hypertonic saline (n = 14 warm, 11 cold). Mean ± one s.e.m.

85.6 ± 1.3% vs. −66.1 ± 2.2% in the cold acclimated fish (Fig. 1 C, 2-way ANOVA *P* values clonidine <0.0001, temperature < 0.05, interaction <0.02), a significantly smaller percent decrease in cold acclimated fish (Mann Whitney *U* test, $P < 0.05$). The blunting of the clonidine response in the cold acclimated fish, compared to the warm acclimated fish, can be readily observed in the full dose-response (Fig. 2C) and the ED_{50} is significantly higher by 1.96-fold in the cold acclimated fish (Fig. 3A; $P = 0.0002$, unpaired *t*-test,). In contrast, there was no difference in the ED_{50} for isoproterenol (Fig. 3B) between the opercular epithelia of warm and cold acclimated fish (Unpaired *t*-test, $P = 0.9031$).

3.1.2. Hypotonicity and hypertonicity

Hypotonicity significantly inhibited I_{sc} in warm and cold acclimated fish (Fig. 1A, 2-way ANOVA *P* values hypotonicity <0.0002, temperature <0.0001, interaction <0.0001) but the effect in warm acclimated fish was more profound, with I_{sc} decreasing −95.0 ± 1.8%, compared to −75.1 ± 2.4% in the cold acclimated fish, mostly attributable to the higher resting level of I_{sc} in the warm acclimated fish. The large statistical interaction reflects the well-known inhibitory effect of low temperature on I_{sc}. Hypertonicity significantly increased I_{sc} in both groups, +4.98-fold in warm and +2.55-fold in the cold acclimated fish (Fig. 1D 2-way ANOVA *P* values hypertonicity <0.0002, temperature NS, interaction NS). The lack of temperature and interaction effect implies that the hypertonicity effect is independent of acclimation temperature. In the warm acclimated fish, the I_{sc} was more variable with some tissues responding poorly but the level of recovery was the same final I_{sc} as the cold acclimated fish and significantly lower than the resting I_{sc} seen before clonidine addition (Fig. 1C). The hypotonicity dose-response (Fig. 2A) indicated that the warm acclimated tissues were inhibited more (−95%) than the cold acclimated fish (−75%). The hypotonic response ED_{50}, expressed as the average decrease in osmolality needed to produce 50% of the total inhibition was significantly lower in cold acclimated fish, −37.9 ± 0.9 $mOsm \cdot kg^{-1}$ (n = 10) compared to warm acclimated fish −44.9 ± 0.6 $mOsm \cdot kg^{-1}$ (n = 13) ($P < 0.0001$, unpaired two tailed *t*-test), suggesting that the cold acclimated animals were more sensitive to osmolality decrease. In contrast, the hypertonic stimulation of I_{sc} was present in both cold and warm acclimated fish (Figs. 1D and 2D) and virtually superimposable (Fig. 2D) with no apparent effect of acclimation temperature.

3.2. Plasma cortisol and osmolality

Plasma cortisol was the same in cold acclimated fish (4.65 ± 1.6 $ng \cdot mL^{-1}$, n = 13) as in warm acclimated animals (6.3 ± 2.8 $ng \cdot mL^{-1}$, n = 13; $P = 0.6116$, unpaired two tailed *t*-test) and was <10 $ng \cdot mL^{-1}$, indicating that cold acclimation does not produce long term stress. However, the cold acclimated animals had significantly elevated plasma osmolality, compared to warm acclimated controls; 419.4 ± 6.0 $mOsm \cdot kg^{-1}$ (n = 14) versus 383.3 ± 3.9 $mOsm \cdot kg^{-1}$ (n = 15), respectively, ($P < 0.0001$, unpaired two tailed *t*-test).

3.3. Mitochondria

The mitochondrial density, width and cristae structure in opercular epithelium ionocytes were unremarkable and not apparently different between cold acclimated and warm acclimated fish. There appeared to be a shape change, in that the profile length of mitochondria in cold-acclimated fish (1.55 ± 0.04 μm) were shorter by 11% than those from warm acclimated fish (1.72 ± 0.04 μm; $P = 0.008$ unpaired two tailed *t*-test n = 18 per treatment).

4. Discussion

The main finding is that while the adrenergic responses were blunted in the cold acclimated animals, the osmotic responsiveness of salt secretion, particularly augmentation by hypertonicity, were as effective in the cold as in warm acclimated animals.

4.1. Osmotic regulation of ion transport

The average response curves show that cold acclimated fish respond to hypertonicity almost identically to warm acclimated animals and in 2-way ANOVA, the effect of temperature was not significant. Hypertonicity restored I_{sc} to the same as initial level (before clonidine addition) in cold acclimated fish, while not restoring transport to the initial level in warm acclimated fish. The relatively enhanced response to hypertonicity in cold acclimated animals suggests that hypertonicity may become a crucial regulator in cold temperatures and may be less important in warm acclimated fish. The hypotonic dose-response revealed an ED_{50} for cold acclimated fish that was lower than for warm acclimated animals, suggesting greater sensitivity in the cold, but the size of the response in the cold was smaller, apparently connected to the lower resting transport rate in the cold.

Overall, the osmotic pathway, tested by exposing the opercular epithelia to hypertonic and hypotonic shock, appears to be more robust than the neural pathway in the cold. The osmotic response is a locally acting pathway that does not require input from the nervous or endocrine systems. The hypotonic response that inhibits ion secretion appears to rely on tightly colocalized phosphorylation components. The coimmunoprecipitations of the osmosensor integrin β1, the tyrosine kinase focal adhesion kinase and the NKCC1 symport provide evidence for a metabolon where regulation of NKCC requires neither receptor interactions nor second messengers (Marshall et al., 2008, Marshall et al., 2009). Mummichogs also appear to experience nervous system depression in the cold, exhibiting limited activity and lower food consumption at low temperatures (Barnes et al., 2014). Similar

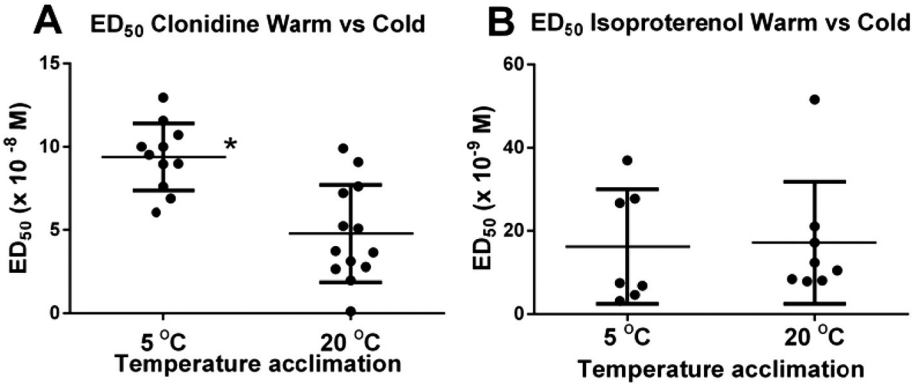

Fig. 3. ED_{50} for inhibition of I_{sc} by clonidine (n = 11 cold, 14 warm) and stimulation of I_{sc} by isoproterenol (n = 7 cold, 8 warm) for opercular epithelia from cold and warm acclimated mummichogs. Means ± one s.e.m. with individual results included, asterisk indicates $P = 0.002$, unpaired two tailed *t*-test.

inactivity and low food intake is seen in Atlantic salmon smolts placed in low temperature seawater (Arnesen et al., 1998).

4.2. Adrenergic regulation of ion transport

The adrenergic pathway, however, stimulates transport by the binding of a neurotransmitter to a receptor and initiates a more complicated cascade of intracellular events than the direct mechanical initiation of the osmotic pathway (O'Dowd et al., 1989). The effective dose at 50% (ED_{50}) is the dose of drug required to produce 50% inhibition of I_{sc}, and is taken as an indicator of the responsiveness of the pathway. Because there was no difference between the ED_{50} value for isoproterenol in warm versus cold acclimated fish, the ion transport is equally responsive to the β-adrenergic pathway in cold and warm acclimated fish. However, the β-adrenergic excitatory pathway is not the dominant pathway when non-selective agonists, such as (the physiologically relevant) adrenaline and noradrenaline, are applied to the ionocyte NaCl secretion systems, the dominant response is inhibition of I_{sc} (May and Degnan, 1985; Marshall, 2003). Hence the dominant α_2-adrenergic pathway is the more sensitive in physiological terms and acts as a means to rapidly inhibit salt secretion when the need arises, for instance during the early phases of acclimation to freshwater. The effects of the α_2-adrenergic agonists thus are the more physiologically important.

The ED_{50} value for clonidine was significantly higher, and the percent inhibition significantly lower in cold acclimated fish than warm acclimated fish, revealing the α-adrenergic pathway to be less responsive in the cold. If down-regulation of α_2-adrenoceptors, the adrenergic pathway may not be able to respond to normal levels of the agonist, resulting in the reduced responsiveness we found, but this should be confirmed by direct measurement of receptor concentration. If mummichogs relied solely on the α_2-adrenergic pathway to inhibit ion salt secretion, they may not be able to retain plasma ions efficiently in freshwater in winter. Under these conditions, the osmotic pathway may become more critical to survival. The α_2-adrenergic pathway may be the primary down-regulator in warm temperatures, whereas the osmotic pathway can become the primary regulatory pathway when the responsiveness of the α_2-adrenergic pathway is diminished in the cold.

Cold acclimation has variable effects on adrenergic responsiveness. In rainbow trout acclimated to cold (8 °C) and warm (18 °C) freshwater, heart power ED_{50} for output and ventricular strip contractility were higher in cold acclimated fish, leading to the conclusion that, in the cold, trout heart has a greater density of cell surface adrenoceptor available for binding (Keen et al., 1993). This result was confirmed with 4 °C vs. 17 °C acclimation temperatures, β-adrenergic inotropic response to heart rate was higher in the cold (Aho and Vornanen, 2001). Analysis of surface expression of β-adrenergic receptors confirmed that there were more β-adrenergic receptors and higher effective cAMP concentrations in the cold, inferring that modulation of receptor number is the primary acclimation response (McKinley and Hazel, 2000). In contrast, steelhead trout (acclimated to 1, 5 and 10 °C) coronary arteries in vitro instead have reduced responsiveness to β-adrenergic epinephrine mediated dilation (also to serotonin and adenosine) but increased sensitivity to cholinergic-mediated constriction (Costa et al., 2015). In carp (*Cyprinus carpio*) acclimated to 5, 10 and 15 °C, cholinergic-mediated hypotension was attenuated by an up-regulation of an α-adrenergically mediated peripheral vasoconstriction during hypoxia (Stecyk and Farrell, 2006). In a detailed species comparison study of adrenergic/cholinergic responses in branchial arteries of temperate and polar species, the authors concluded that there is little effect of environmental temperature, latitude, or lifestyle on the pattern of receptor-mediated vascular control (Hill and Egginton, 2010). Finally, some Antarctic fishes seem to have virtually eliminated adrenergic control over heart rate but instead reduce cholinergic-mediated tonus to produce tachycardia (Davison et al., 1997). Whereas most research has focused on circulatory acclimation to cold, there is one study on liver glycogenolysis responses in the cold. In rainbow trout hepatocytes, acclimation to cold (5 °C vs. warm 20 °C), both basal and epinephrine-stimulated rates of glucose release were higher in 5 °C and these results remained regardless of perfusion temperature (McKinley and Hazel, 1993).

Thus the literature on heart, blood vessels and hepatocytes reveals much interspecies variation of β-adrenergic regulation of heart rate, vessel tone and glucose mobilization in acclimation to cold conditions. Among the studies of adrenergic responses of fish in the cold, our study is the only one to examine ion transport and only the second to focus on α-adrenergic responses (Hill and Egginton, 2010). It would seem that down-regulation of α-adrenergic control of ion transport, where alternate osmotic controls exist, would be energetically efficient and could contribute to survival in the cold.

4.3. Mitochondrial adaptation in the cold

Because the stress of cold acclimation slows down biochemical processes, mitochondria have been documented to respond in several different ways. In our study, apparently the first to examine mitochondria of salt secreting ionocytes in the cold, mitochondria appeared to be in similar densities and with similar cristae structure in warm (20 °C) and cold (5 °C) acclimated fish, but section profile length of mitochondria was significantly reduced in the cold, indicative of a compensatory response, perhaps explained by an increase in tortuosity of mitochondrial shape. In skeletal muscle of cold (5 °C) acclimated striped bass (*Morone saxatilis*), mitochondrial hypertrophy increases surface area of mitochondria, especially in glycolytic fibers, without change in surface density of cristae (Egginton and Sidell, 1989). In cold (1 °C) acclimated rainbow trout (*Oncorhynchus mykiss*) skeletal muscle cristae surface area increases significantly (St-Pierre et al., 1998). Also in rainbow trout skeletal muscle, cold (4 °C) acclimation increased total mitochondrial surface area and capillary density, such that predicted O_2 delivery to swimming muscles is the same winter and summer. Muscle mitochondria of cold acclimated rainbow trout have higher volume density in winter, but apparently similar structure (Egginton et al., 2000). The large mitochondrial hypertrophy and sometimes also the increase in cristae area in cold skeletal muscle likely helps increase ATP supply in winter conditions, but the response may be tissue specific, as the gill ionocytes do not respond similarly and the relatively subtle change we observed in mitochondrial shape has not been observed previously.

4.4. Cortisol and cold acclimation

Cold shock has been well studied and cortisol level is well recognized as a sensitive indicator of stresses, including cold stress (review: Donaldson et al., 2008). Because mummichogs are strongly eurythermic (Fangue et al., 2009), we anticipated that cold acclimation would result in no stress response and the very low measured level (4.6 ± 1.6 $ng \cdot mL^{-1}$) demonstrates that the animals were not stressed by winter conditions (5 °C). Stenothermic fish instead may enter a chronically stressed condition in the cold. Sea bream (*Pagrus major*), reared at 7 °C, as compared to 10 and 13 °C, have markedly elevated plasma cortisol and glucose even after 15 days in the cold (Hwang et al., 2012) and sea bream is subject to long term stress and mortality from 'winter syndrome' (Ibarz et al., 2007). In contrast, eurythermic temperate fish can endure rapid cooling, for instance Atlantic salmon can be cooled rapidly from 16 °C to 8 °C or 4 °C and not have elevated cortisol levels (Foss et al., 2012). The unner (*Tautogolabrus adspersus*), a cold water living temperate fish, has modestly higher than resting cortisol levels at 0 °C ($15\ ng \cdot mL^{-1}$), well below an active stress level, but these fish have a blunted stress response (to 1 min air exposure), compared to control fish at 10 °C (Alzaid et al., 2015). For fish such as the mummichog that

must overwinter under ice, eurythermy is well-developed and living in cold conditions is not stressful.

4.5. Conclusions and future directions

Hypertonic shock was a very effective stimulant in cold acclimated fish, increasing I_{sc} to higher than initial levels (before clonidine addition) in the cold. We also found that clonidine had a significantly higher ED_{50} and lower maximal inhibition in cold acclimated fish, meaning the α_2-adrenergic pathway is less responsive in the cold. These two results suggest that the osmotic pathway is the more reliable regulator of ion transport in cold acclimated fish, likely because it is a locally controlled pathway that does not require receptor-hormone interactions or diffusible second messengers.

The present study illustrates how the functionality of ion transport regulatory pathways at the molecular level can affect cold tolerance at the organismal level. Because of the simplicity and efficiency of the osmotic pathway, it operates at normal levels in the cold, whereas the potentially less efficient diffusible secondary messengers and more complicated hormone receptors hinder the adrenergic pathway. The functionality of the osmotic pathway in the cold at a molecular level thus increases the capacity for cold tolerance at the level of the whole organism.

These results highlight the need for control of critical physiological processes by multiple, apparently redundant, pathways in response to extreme physiological stressors such as the cold. This study also provides insight into how eurythermic species such as the mummichog maintain ion transport regulation in the cold, and how they can survive low temperature extremes to overwinter in northern waters.

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