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The Influence of Polyamines on the Hypotonic Shock Response in the Euryhaline Teleost, Fundulus grandis

Keith Lasko

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by

Keith Lasko

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Dr. Galvez

Department of Biological Sciences

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Louisiana State University
& Agricultural and Mechanical College
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Abstract

The ability of the gulf killifish, *Fundulus grandis*, to acclimate to a wide range of salinities makes it a model organism for the study of osmoregulation. *Fundulus grandis* is able to rapidly acclimate to short-term freshwater exposure through a sympathetic-mediated hypotonic shock reflex that minimizes salt secretion at the gills. It is known that hypotonic shock elevates the transcription rate of polyamine synthesizing enzymes. This study uses electrophysiology techniques to determine the role of polyamines in the osmoregulation of killifish during acute transfer to hypotonic media. Cl^- secretion is measured indirectly as I_{sc} (short-circuit current) using the voltage-clamp method. By applying Ohm's Law, R_t (transepithelial resistance) can be easily calculated as well. Epithelia treated with polyamines at the moment of hypotonic exposure demonstrated a rapid reduction in Cl^- secretion that was significantly greater than the reduction experienced by the control group. However, once steady state was reached under hypotonic conditions, the treatment group inhibited Cl^- secretion to a lesser degree compared to the control group. The polyamine treatment was also shown to inhibit the increase in R_t that occurs under hypotonic conditions. These findings support the theory that polyamines contribute to short-term acclimation during hypotonic exposure.

Introduction

Gulf killifish, *Fundulus grandis*, reside primarily in estuaries and marshes along the Atlantic coast of Florida and along the coast of the Gulf of Mexico (Robins and Ray 1986). *Fundulus grandis* can survive acute exposure to salinities as high as 35 g/L after acclimation to 3 g/L (Crego and Peterson 1997). The physiological plasticity of *Fundulus grandis* in response to changes in temperature, dissolved oxygen, and salinity, and its relative abundance in the wild makes it a model organism for the study of ionoregulatory responses to osmotic stress (Wood

and Marshall 1994). Killifish acclimated to saltwater are able cope with short-duration freshwater exposure by regulating salt secretion through reversible remodeling of the fish gill epithelium (Marshall 2003). This rapid morphological change is mediated by a hypotonic shock reflex. Genes involved in the transcription of arginase and orthinine decarboxylase, enzymes responsible for the biosynthesis of polyamines, are highly upregulated during hypotonic shock (Whitehead et al. 2012). The role of polyamines during hypotonic stress is currently unknown and is the main focus of this paper. I suspect that polyamines facilitate an inhibition of Cl^- secretion by downregulating one or more of the integral membrane proteins associated with an efflux of NaCl at the fish gill epithelium. Polyamines may also facilitate the physical covering of chloride secreting cells by pavement cells.

Gross Anatomy: Teleost fish have evolved a flap of body wall supported by bone, known as an operculum, which extends from the hyoid arch region of the head laterally and caudally over the gills. There is one large opercular cavity that houses all the gills and one valved, external gill slit (Homberger and Walker 2003). The opercular system produces an uninterrupted, unidirectional flow of respiratory water from the oropharynx, through the gills, and out through the opercular cavity. This system provides optimal countercurrent exchange with oxygen-poor blood, originating from afferent branchial arteries, at the capillaries within the gill lamellae (Liem et al. 2001). The gill epithelium is a physical boundary between a fish's external environment and extracellular fluid. Specialized epithelial cells, known as ionocytes, are found predominantly in the gill epithelium of teleost fish and are important for the maintenance of osmotic homeostasis (Hiroi and McCormick 2012).

The gill epithelium primarily consists of pavement cells (PVCs) and mitochondrion-rich cells (MRCs), which comprise about 89% and 9% of the epithelial surface area respectively

(Evans 2005). The opercular epithelium is a good surrogate of the gill epithelium in vitro in that it has a high density of MRCs and can also be mounted in an Ussing Chamber so that ion transport can be measured (Zadunaisky 1996). It is frequently used to study the mechanisms of cell volume regulation during osmotic challenges (Marshall 2005).

An Ussing chamber system consists of two compartments that are separated by the epithelium of interest, isolating the apical (or mucosal) side of the membrane from the basolateral (or serosal) side. Each compartment is filled with equal amounts of identical electrolyte solutions, eliminating osmotic and hydrostatic gradients that would otherwise influence the movement of ions across the epithelium. Ion transport across the epithelium generates a voltage difference that is measured by two voltage electrodes. This voltage difference is artificially clamped to zero by two current electrodes that inject current to equally oppose the voltage generated by the epithelium. This short-circuit current (I_{sc}) is a measure of the net ion transport across the epithelium (Clarke 2009).

Anatomy of Opercular Epithelium: Superficial pavement cells are the most abundant cell type covering the epithelium. They are squamous to cuboid-shaped cells with a low density of mitochondria and have distinct microridges or microvilli on the apical surface (Laurent 1984, Olson 1996). Pavement cells are joined together by desmosomes. The tight junctions associating pavement cells with MRCs have multiple strands and are presumed to be impermeable to ions (Sardet 1979, Kawahara 1982). Cellular hypotonic shock incurs a sympathetic neural reflex that results in the covering of MRCs by PVCs (Marshall 2003). Although PVCs were previously believed to be morphologically unresponsive to environmental changes (Laurent 1984), it is possible that scientists may have underestimated the importance of PVCs in favor of studying MRC's role in ion transport. The apical surface area of PVCs has been shown to increase in

response to high levels of blood CO_2 (Goss et al. 1994). Furthermore, TEM examination of catfish gill PVCs show studded cytoplasmic vesicles and apical crypts resembling proton-secretory epithelia (Laurent et al. 1994).

Although MRCs are relatively few in number on the surface of gill and opercular epithelia, they are the primary ionocytes in regards to fish osmoregulation. They are highly susceptible to phenotypic change during environmental changes in salinity (Evans et al. 2005). The high density of MRCs found in the opercular epithelium of gulf killifish are structurally and functionally identical to those found in the gill epithelium in saltwater fish (Karnaky and Kinter 1977, Zadunaisky et al. 1995). MRCs of saltwater teleost are characterized by elaborate intracellular systems of branching tubules that are continuous with the basolateral membrane. This tubulovesicular system is closely associated with mitochondria that directly supply basolateral Na^+K^+ATPase anitorters with ATP for primary active transport (Evans et al. 2005).

The gill epithelia of saltwater-type gulf killifish contain two distinct types of MRCs: chloride cells (CCs) and accessory cells (ACs). The term MRC is often used interchangeably with chloride cell even though accessory cells are also characterized by a high density of mitochondria (Evans 2005). Accessory cells, relative to chloride cells, are much smaller, have a less defined tubulovesicular system, and have lower expression of basolateral Na^+K^+ATPase antiporters (Hootman and Philpott 1980). Chloride cells and accessory cells are in close association with each other. Their apical regions form deep invaginations known as apical crypts. Active Cl^- secretion into the apical crypt induces a positive apical membrane potential. This potential facilitates passive Na^+ secretion via a leaky paracellular pathway that exists between accessory cells and chloride cells (Marshall 2010).

Salt-transporting integral membrane proteins: The gill epithelium possesses a suite of transporters responsible for salt secretion in hypertonic environments. The complexity and physical frailty of the fish gill necessitates several indirect experimental approaches and many of its features are still poorly understood. The current proposed model of salt secretion suggests that Na^+K^+ATPase antiporters on the basolateral membrane of chloride cells is responsible for generating a low intracellular Na^+ concentration. Administration of the Na^+K^+ATPase inhibitor, ouabain, into the bloodstream of saltwater eels results in total inhibition of salt secretion at the gills (Silva et al. 1977). This transepithelial gradient facilitates the secondary active transport of Cl^- into the cytosol through a basolateral NKCC1 symporter. Sensitivity of Cl^- secretion to furosemide, a NKCC inhibitor, and insensitivity to thiazide, a Na^+Cl^- cotransport inhibitor, suggests that the basolateral carrier belongs to the NKCC-type transporter family (Payne and Forbush 1995). Once in the cytosol of the chloride cell, Cl^- passively diffuses across the apical membrane where the anion channel CFTR conducts it into the apical crypt. This Cl^- channel is stimulated by cAMP and is insensitive to 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), an anion exchange inhibitor, suggesting that it is homologous with human Cystic Fibrosis Transepithelial Conductance Regulator (CFTR) (Marshall et al. 1995). The positive intracellular electrical gradient generated by Cl^- efflux drives Na^+ passively out through shallow, tight junctions between chloride cells and accessory cells via the paracellular pathway.

An examination of epithelial cells experiencing hypotonic shock under scanning electron microscopy (SEM) revealed a 23% decrease in apical crypt density compared to the epithelia of fish acclimated to salt water (Daborn and Marshall 2001). These findings imply the active blocking of apical crypts by pavement cells, effectively decreasing ion permeability at chloride cells. Although the covering of ionocytes by pavement cells in hypotonic media is consistent

with an increase in transepithelial resistance (R_t), a decrease in the flow of current (I_{sc}) precedes this, suggesting that the rate of ion secretion is inhibited before the apical crypts are covered (Marshall 2000).

The actin-based cytoskeleton is implicated in volume-sensing and regulation in a wide range of cells and tissues (Pederson et al. 1999, Hoffmann and Mills 1999). Although the exact mechanism has yet to be identified, the current hypothetical model for rapid regulation of Cl^- secretion involves actin-mediated phosphorylation and dephosphorylation events on integral membrane proteins during regulatory volume increase (RVI) and regulatory volume decrease (RVD), respectively. Cell swelling from hypotonic shock may cause actin-activation of a phosphatase that, in turn, downregulates basolateral NKCC. This is proven to be the case in shark rectal gland cells, which bear many similarities to gill epithelial cells (Darman et al. 2001). This hypothesis is further complemented by the fact that calyculin A, a serine/threonine protein phosphatase inhibitor, stimulates NKCC1 in Ehrlich cells (Krarup et al. 1998). Cytochalasin D, an actin disrupter, prevents cAMP-dependent phosphorylation of CFTR, illustrating a likely connection between the actin cytoskeleton and the upregulation of CFTR (Prat et al. 1995). It is postulated that polyamines, particularly putrescine, may play a role in the activation of second messenger proteins that facilitate the short-term inactivation of Na^+K^+ATPase during acute cell swelling (Watts 1996).

Polyamine synthesis amid osmotic stress: Polyamines serve a wide array of functions in vertebrates, including cell-cell communication, apoptosis, regulation of gene expression, and cell growth (Ree et al. 2007). It has been suggested that polyamines may be a vital component in gill remodeling or to regulating cell volume (Laurent et al. 2006) in euryhaline teleost fish. The biosynthesis of polyamines is dependent on two major enzymes, arginase and ornithine

decarboxylase. Arginase catalyzes the hydrolysis of arginine to ornithine and urea (DiCostanzo et al. 2007). Ornithine decarboxylase, the rate limiting enzyme of polyamine synthesis, catalyzes the decarboxylation of ornithine to putrescine, from which spermidine and spermine are derived (Kern et al. 1999). This study seeks to determine the direct influence of polyamines on salt secretion and transepithelial resistance at the gill epithelium of saltwater-acclimated *Fundulus grandis* during hypotonic exposure.

Materials and Methods

Adult gulf killifish (*Fundulus grandis*) were collected in Cocodrie, Louisiana near Louisiana Universities Marine Consortium (LUMCON). Specimens were then transferred to an indoor aquatic facility in the Life Sciences Building at Louisiana State University and acclimated to 12 ppt salinity at 22°C. Fish were held in artificially formulated seawater made by dissolving Instant Ocean™ brand sea salts into water purified by reverse osmosis. This water was derived from de-chlorinated tap water in a Culligan Aqua-Cleer® water treatment system. Water quality was assessed twice a week with the YSI Pro 2030 water quality meter. Dissolved oxygen was held to approximately 8 ppm. Salinity was held to approximately 12 ppt. Temperature was held to approximately 22 °C. pH was measured twice a week on a Denver Instrument UltraBasic UB-10 pH/mV meter and held to approximately 7.5. Total nitrite, nitrate and ammonia were measured daily using API Aquarium Pharmaceutical Water Chemistry test kits. Ammonia and nitrate were both held to less than 1 ppm and nitrate was held to less than 20 ppm. Water exchanges were performed twice a week and fish were fed once daily with 3.17 mm Cargill brand pellet food.

Gulf killifish were weighed prior to being anesthetized in 0.2 g/L of Tricaine methanesulfonate (MS-222) for 6 minutes and euthanized by cervical dislocation at the junction

between the skull and the first trunk vertebra. The paired opercular epithelia were dissected from the medial surface of the left and right opercular bones with liberal application of isotonic bathing solution (described below) and mounted between two plexiglass sliders (P2403. Physiologial Instruments Inc.) with an exposed area of 0.1 cm^2. These sliders were then placed in individual Ussing Chambers (EM-CSYS-4, Physiological Instruments Inc.). The basolateral side of the membrane was bathed in 3 ml isotonic buffer solution with added 3 mM Glucose. The apical side of the membrane was bathed in only 3 ml isotonic buffer solution.

The isotonic bathing solution (334 mosm/L, pH 7.8) was used to prevent desiccation of the opercula during dissection and also to bathe both membrane surfaces symmetrically under conditions that mimic seawater. The composition of the isotonic buffer was, in mmol/L: 151 NaCl, 3.0 KCl, 0.88 MgSO4, 4.6 Na2HPO4, 0.48 K2HPO4, 1.0 Cacl2, 11 HEPES, 4.5 Urea. The buffer was then bubbled for 5 minutes with 100% O2 gas. The hypotonic buffer (270 mosm/L, pH 7.8) was made by diluting the isotonic buffer with deionized water (75% isotonic buffer and 25% deionized water) and was then bubbled for 5 minutes with 100% O2 gas. The negligible change in fluid resistance as a result of the dilution was not compensated for during experimentation.

It has been shown using the vibrating microprobe extracellular recording technique that I_{sc} (short-circuit current) and R_t (transepithelial resistance) directly correlate with Cl^- secretion at the apical surface of chloride cells, a type of mitochondrion-rich cell (Foskett and Machen 1985). I_{sc} was measured in real time with data acquisitions software (PowerLab and LabChart 7 software, ADInstrument Inc). The raw data did not take into account the surface area exposed on the plexiglass aperture (0.1 cm^2). Therefore the true I_{sc} ($\mu A/cm^2$) is one order of magnitude greater than the raw data implies. Since voltage is artificially held to a constant by two current

electrodes, any change in membrane flux that occurs as a result of chloride secretion is detected as the amount of I_{sc} that must be artificially injected to return the voltage to the predetermined constant. V_t (transepithelial voltage) is the difference between the voltage at the apical side of the membrane and the voltage at the basolateral side of the membrane. This reflects the asymmetric distribution of ion channels between the two sides of the membrane. V_t (mV) was periodically measured in open circuit. R_t ($\mu\Omega \cdot cm^2$) was calculated by applying Ohm's Law, V=IR. Voltage asymmetries across the electrodes were zeroed and fluid resistance was compensated for prior to experimentation. The circuit was clamped to 0 mV and a pulse of 2 mV was applied every minute by a pulse generator so that R_t could be calculated from the equation: $R_t = 2000(\mu V)/ \Delta I_{sc}(\mu A/cm^2)$. To determine V_t, the feedback circuitry was periodically set to the open circuit condition.

The opercula were bathed in isotonic buffer under symmetrical conditions until a steady state was reached after 30-40 minutes. For the control experiment, the hypotonic shock reflex was induced by replacing the isotonic buffer with 3 ml hypotonic buffer and allowed to reach steady state after 20-30 minutes. To determine the effects of polyamines on chloride secretion during hypotonic shock, the treatment group was supplemented with 0.1 mM spermine, 0.4 mM putrescine, and 0.3 mM spermidine on the basolateral side of the membrane as the transfer from isotonic buffer solution to hypotonic buffer solution was made. It has previously been demonstrated that larger concentrations of polyamines do not elicit a stronger response in the opercular epithelia of *Fundulus grandis* (Guan 2013).

Results

Experiments were performed on seven opercula from five male adult fish (weight range of 8.4 g to 13.2 g). Four opercula from four different fish are included in the control group. Three

opercula from three different fish are included in the treatment group. Data are expressed as a mean ± 1 unit of standard deviation. With or without polyamine treatment, it took roughly half the time for steady state to be reached in hypotonic media (20 minutes) compared with isotonic media (45 minutes). V_t (transepithelial voltage) proved unaffected by hypotonic shock (data not shown). In the control group (figure 1A), a 67 mosm/L decrease in osmolarity was accompanied by an immediate reduction in I_{sc} from 22.03 $\mu A/cm^2$ to 7.90 $\mu A/cm^2$, a 64% reduction in I_{sc}. Thirty minutes after transfer, I_{sc} declined further to 5.40 $\mu A/cm^2$, a 76% reduction in I_{sc}. For the treatment group (figure 1B), the transfer to hypotonic media elicited a reduction in I_{sc} by 99%, from 22.38 $\mu A/cm^2$ to 0.06 $\mu A/cm^2$. This reduction was followed by a gradual recovery of I_{sc}. Thirty minutes after transfer, I_{sc} had increased to 10.98 $\mu A/cm^2$, which is a 32% reduction compared to the I_{sc} of the opercula in isotonic saline.

R_t (transepithelial resistance) was measured once when the opercular epithelia had come to a steady state in isotonic media and then measured once again for comparison upon reaching a steady state in hypotonic media. Hypotonic shock in the control group (figure 2A) was shown to increase R_t from 32.8 $\mu\Omega \cdot cm^2$ to 62.1 $\mu\Omega \cdot cm^2$, a 66% increase. This event was restricted to a 30% increase, from 34.3 $\mu\Omega \cdot cm^2$ to 44.64 $\mu\Omega \cdot cm^2$ in the treatment group (figure 2B). In other words, ion conductance across the opercular epithelium decreases amid hypotonic shock to a lesser extent in the presence of polyamines.

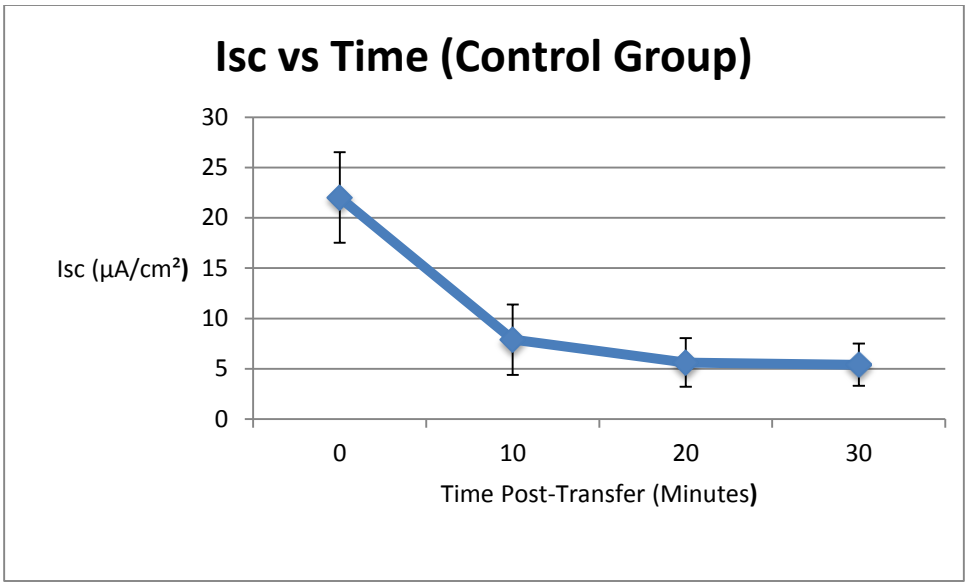
Discussion

The ability of *Fundulus grandis* to arrest salt secretion at gill ionocytes is critical to its viability in dilute media. The rapid inhibition of Cl^- secretion in response to catecholamines suggests that the hypotonic shock reflex is mediated by the sympathetic nervous system. Marshall and his colleagues demonstrated that stimulation of the glossopharyngeal nerve (cranial

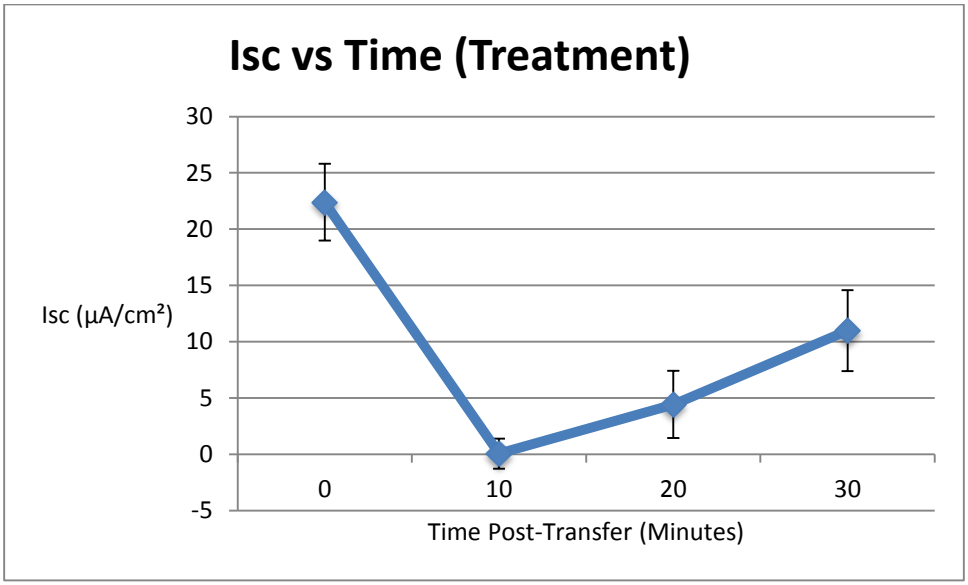
nerve IX) innervating an opercular epithelium immediately reduced I_{sc} by 30% (Marshall et al. 1998). The fact that this effect was blocked by yohimbine suggests that catecholamines are primarily acting on α_2 adrenergic receptors on the gill epithelium (May et al. 1984). A greater affinity for norepinephrine as opposed to epinephrine by the gill epithelium is another characteristic of α_2 adrenergic receptors (Marshall et al. 1993). α_2 adrenergic receptors are coupled to adenylyl cyclase by a G-inhibitory protein. The binding of a catecholamine inhibits adenylyl cyclase, ultimately decreasing intracellular levels of cAMP (Costanzo 2014). Experimentally increasing cAMP levels in killifish opercular membranes facilitates an increase in apical Cl^- conductance (May and Degnan 1985). It can be postulated that CFTR is regulated by a cAMP-dependent kinase. Inhibition of Cl^- secretion would reduce the serosa positive potential and inhibit Na^+ secretion. Stimulation of the sympathetic nervous system did not completely inhibit Cl^- efflux; therefore other mechanisms must contribute to immediate freshwater acclimation.

Levels of putrescine were shown to be inversely proportional to Na^+K^+ATPase activity in nauplii (crustacean larvae) acclimated to various salinities in vitro (Lee 1992). Conversely, although transcription of polyamine synthesizing enzymes is upregulated during hypotonic shock, putrescine concentrations in the saltwater killifish gill remain low until 24 hours after transfer to freshwater (Munley et al. 2014). Concentrations of spermine and spermidine in fish gills must be measured next to verify whether or not low putrescine levels are caused by its conversion to spermine and spermidine. The killifish epithelia treated with a polyamine mixture on the basolateral side underwent an immediate and dramatic reduction in I_{sc} under hypotonic shock. This finding supports the theory that polyamines contribute to short-term acclimation during hypotonic exposure. Paradoxically, the treatment group maintained a significantly higher

I_{sc} than the control group once steady state was reached in hypotonic media. These findings might suggest that polyamines are detrimental to killifish viability during long-term hypotonic exposure. Another possible explanation for this paradox is that increased Cl^- secretion is a compensatory mechanism of regulatory volume decrease (RVD) that alleviates cell swelling caused by an osmotic pressure gradient. R_t increase during hypotonic shock is mostly due to the covering of apical crypts by pavement cells. My findings that polyamines inhibit R_t increase contradicts the hypothesis that polyamines facilitate the covering of apical crypts. The physiological effects of polyamines on osmotic stress tolerance in euryhaline teleost remain enigmatic. Information to be gained on the subject via Ussing chamber experimentation is limited and other experimental methods must be explored.



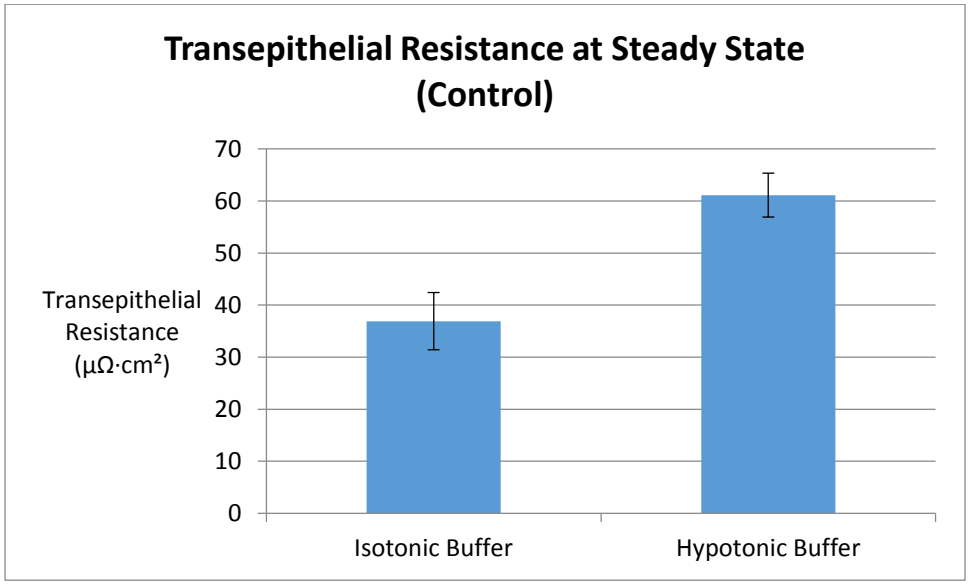
(Figure 1A)



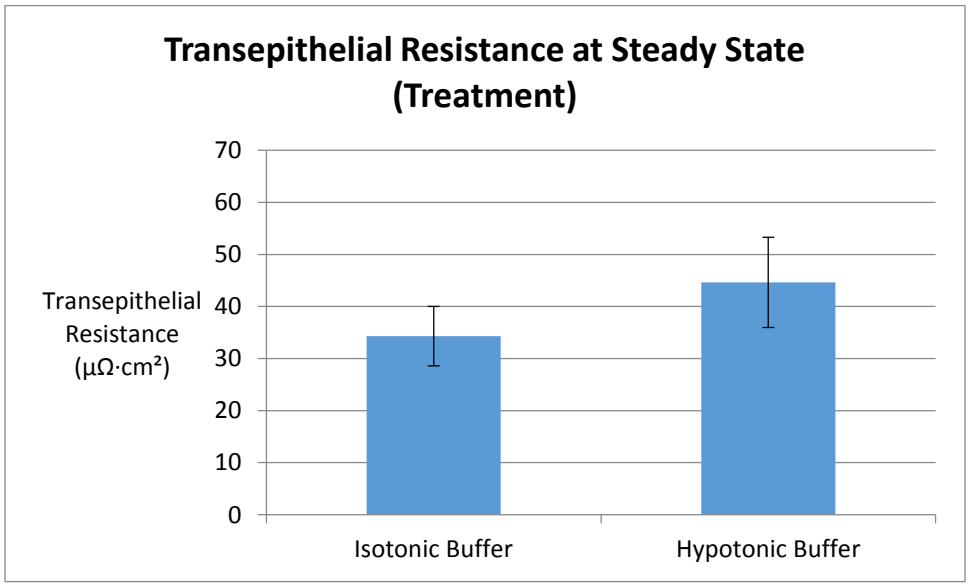
(Figure 1B)

Figure 1: Data represents the mean I_{sc} (Short-circuit current) ± 1 S.D. of *Fundulus grandis* opercular epithelia mounted in an Ussing Chamber after transfer from isotonic buffer (334 mosm/L) to hypotonic buffer (270 mosm/L). Figure 1A represents those epithelia (N=4) that did not receive supplementation with a polyamine mixture at the moment of transfer to the hypotonic medium. Figure 1B represents those epithelia (N=3) that were supplemented with a polyamine

mixture (0.4 mM putrescine, 0.3 mM spermidine, and 0.1 mM spermine) on the basolateral side of the membrane at the moment of transfer to the hypotonic medium.



(Figure 2A)



(Figure 2B)

Figure 2: Data represents the mean value of R_t (Transepithelial Resistance) ± 1 S.D. that was measured once the opercular epithelia had reached steady state under isotonic conditions and then once again when steady state was reached under hypotonic conditions. Figure 2A represents

those epithelia (N=4) that did not receive supplementation with a polyamine mixture at the moment of transfer to the hypotonic medium. Figure 2B represents those epithelia (N=3) that were supplemented with a polyamine mixture (0.4 mM putrescine, 0.3 mM spermidine, and 0.1 mM spermine) on the basolateral side of the membrane at the moment of transfer to the hypotonic medium.

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