The Role of Polyamines in Osmotic Stress Tolerance in Gulf Killifish Fundulus Grandis

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ABSTRACT

The main objective of this study was to describe the mRNA levels and enzymatic activities of key enzymes for polyamine metabolism, to measure polyamine levels, and to assess putative roles of polyamines in the gills of the Fundulus grandis during hypoosmotic challenge. The influence of irreversible inhibition of Odc by alpha-DL-difluoromethylornithine (DFMO) was also assessed in the gills. The mRNA levels and enzymatic activities of Arg, Odc and Pao was assessed in other tissues during hypoosmotic challenges.

Adult F. grandis were reared in 5 ppt and acutely transferred to 5, 2, 1, 0.5, and 0.1 ppt water, and gills were sampled at 6 h, 1 d, 3 d, and 7 d post-transfer. Hypoosmotic exposure produced increases in the arg II and odc mRNA levels, in gill Odc activity, and in the concentrations of putrescine, spermidine, and spermine. DFMO application inhibited Odc activity and reduced polyamine levels after 0.1 ppt transfer. The ratio of putrescine level over the sum levels of spermidine and spermine increased after 0.1 ppt exposure at 1 d and beyond. Concomitant with freshwater acclimation, an increase in Pao activity suggested that polyamine catabolism was upregulated in the gills. The phenotype of mitochondrion-rich cells (MRCs) in the gill epithelium shifted from a seawater type to a freshwater type following transfer to 0.1 ppt water in correlation with the increase in mRNA levels of arg II and odc in MRCs. In addition, the isolated opercular epithelium pretreated with spermidine had a lower active Cl⁻ secretion rate and membrane conductance following symmetrical hypotonic exposure. The mRNA levels and enzymatic activities of Arg II, Odc, and Pao were upregulated in the intestine and liver during hypoosmotic exposure, suggesting that polyamine levels are regulated in
multiple tissues of the killifish. The putative roles of polyamines include inducing cell apoptosis by increasing caspase-3 activity, stimulating cell proliferation by increasing the levels of *c-fos* and *c-myc* mRNA levels, and inducing cell swelling via the modulation of Cl\(^-\) secretion in the gills following hypoosmotic challenges.

In summary, highly cationic polyamines mediate early phase compensatory responses in the euryhaline killifish when faced with osmotic challenges.
CHAPTER 1: LITERATURE REVIEW

1.1 General principles of osmoregulation in teleost fish

Teleost fish regulate the ionic strength and composition of their extracellular fluids (ECF) predominantly by transporting epithelia in organs such as the gills, intestines, and kidneys. In fresh water (FW), the ECF of a fish is hyperosmotic compared to its ambient environment; therefore, a fish must actively absorb ions from water via the gills and produce large amounts of hypoosmotic urine in the kidney to compensate for passive ion loss and osmotic water gain (Evans et al., 2005). In sea water (SW), the ECF of a fish is hypoosmotic compared to its aquatic environment. As a result, SW fish absorb both salt and water from ingested sea water to promote water absorption in the intestine; these salts are subsequently excreted actively at the gills, while the kidney produces small volumes of an isoosmotic urine (Wood and Marshall, 1994).

Fish have varying capacities to expend metabolic resources to fuel active ion transport (i.e., active salt absorption in FW fish or active ion secretion in marine teleosts) (George et al., 2006). Furthermore, fish have varying abilities to transform their transporting epithelia in response to fluctuations in environmental salinity. These differences in energy reserves and in the capacity for physiological plasticity and tolerance to osmotic imbalances define the distribution of a fish in its environment. Most teleost fish tolerate a narrow range of environmental salinities and/or have limited physiological plasticity. In contrast, euryhaline fish are able to maintain the osmotic balance of their ECF over a larger range of environmental salinities. This group of fish can either tolerate osmotic challenges for short periods of time or acclimate to osmotic
challenges for prolonged periods while making compensatory adjustments to the properties of their transporting epithelia.

1.2 Euryhaline Fundulus species – model for environmental salinity tolerance

Some species of fish from the family Fundulus are extremely euryhaline and either transfer between SW and FW quickly and repeatedly (Evans et al., 2005; Marshall, 2003), or acclimate long term to salinities ranging from FW to several times the strength SW.

The gulf killifish $F. \text{grandis}$ and its sister taxa $F. \text{heteroclitus}$ reside in coastal marshes that are subject to frequent and episodic oscillations in salinity, dissolved oxygen, and temperature (Marshall, 2003; Wood and LeMoigne, 1991). Their tolerance to dynamic habitats has made them a widely accepted model for studying the physiological basis of environmental challenges (Burnett et al., 2007). Although tending to reside in brackish to marine salinities (Kaneko and Katoh, 2004; Wood and Grosell, 2008; Wood and Grosell, 2009), killifish are able to tolerate short bouts of freshwater by making rapid, yet minor physiological adjustments (Marshall, 2003; Wood and Grosell, 2008; Wood and Grosell, 2009). However, with prolonged exposure to freshwater, $F. \text{grandis}$ can restore ECF osmotic balance by making much larger compensatory adjustments to the physiology of their ion-transporting epithelia (Wood and Grosell, 2008).

1.3 The influence of environmental salinity on fish gill remodeling

The epithelium that covers the filaments and lamellae of a fish gill separates the ECF from the external environment (Laurent and Dunel, 1980; Evans et al., 2005). This gill epithelium is a highly complex, multicellular organ; however, the pavement cells (PVCs) and the mitochondrion-rich cells (MRCs) are the cell types most often implicated
in osmoregulation. PVCs are thin squamous or cuboidal cells with an extensive apical surface area that are organized in a stratified manner (Wilson and Laurent, 2002). A special sub-population of PVC, the mitochondrion-rich pavement cells (PV-MRC), exists in freshwater-acclimated rainbow trout and is characterized by its irregularly-shaped nucleus, dense peripheral chromatin staining, and absence of a vesicular-tubular network (Galvez et al., 2002; Goss et al., 2001; Laurent et al., 2006). PV-MRCs are also called peanut lectin agglutinin (PNA)-negative MRCs because of their abundance of mitochondria, but their inability to bind peanut-lectin agglutinin (PNA) (Galvez et al., 2002; Goss et al., 2001; Reid et al., 2003). Although PNA immunoreactivity has not been described in killifish gills, analogous PV-MRCs in freshwater-acclimated F. heteroclitus, characterized by their wedge-like appearance at the gill surface between neighboring PVCs, have been termed cuboidal cells (Laurent et al., 2006).

The ultrastructure and function of the gill epithelium, in particular the structure of MRCs, are highly variable with environmental salinity. Despite occupying only a comparably small fraction of the external surface area of the gill, MRCs are essential for fish osmoregulation. The surface of SW-type MRCs is smooth and concave, and underlies an apical crypt guarded by PVCs, as shown in Figure 1.1 panel A. The cell border of MRCs is oval-shaped with poorly developed microvilli towards the center of cells (Katoh et al., 2001). In contrast, the cell surface of FW-type MRCs is irregularly-shaped, often protruding above the surface of the surrounding PVCs and possessing well developed microvilli (Katoh et al., 2001; Katoh and Kaneko, 2003), as shown in Figure 1.1 panel B. Most MRCs have a columnar structure that spans the entire thickness of the epithelium, high mitochondrial density, and abundant expression of transport proteins,
making them particularly effective at transepithelial ion movements (Laurent et al., 1995; Wilson and Laurent, 2002). Though PVCs comprise most of the gill surface, MRCs are thought to be the primary sites for active ion transport (Evans et al., 2005), and presumably more susceptible to phenotypic change during environmental stress. MRCs could physically migrate onto the lamella in order to increase the area for ion uptake after exposure to soft water (Goss et al., 1998; Perry, 1997; Whitehead et al., 2011).

Moreover, the area of apical surface exposure to the environment and the total number of MRCs on the gills are altered by disturbances in acid-base balance, or environmental salinity and oxygen (Claireaux et al., 2004; Dunel-Erb and Laurent, 1980).

Figure 1.1: Scanning electron micrographs on the afferent filament of the gill epithelium from *F. grandis* after 3 d exposure to 5 ppt (A) or 0.1 ppt (B). The arrow indicates an apical crypt of a MRC in a SW-acclimated fish, and the asterisks indicate the apical surfaces of FW-acclimated MRCs.

The gill epithelium has the ability to remodel itself in response to environmental and physiological perturbations. This is well exemplified in the crucian carp (*Carassius carassius*), which can proliferate its interlamellar cell mass (ILCM) of its gill epithelium to the extent of completely covering the gill lamellae within hours of exposure to
normoxia (Sollid et al., 2003). Although the exact mechanism of gill remodeling remains uncertain, low oxygen and high temperature both induce gill remodeling by stimulating apoptosis in the ILCM of crucian carp gill (Sollid and Nilsson, 2006). Moreover, hypoosmotic conditions stimulate apoptosis or necrosis in F. heteroclitus, although there is debate regarding the relative importance of each pathway to remodeling (Katoh and Kaneko, 2003; Laurent et al., 2006). Other stressors, such as heavy metals, also stimulate cellular remodeling of tilapia (Oreochromis, mossambicus) fish gill by altering the relative rates of apoptosis and proliferation (Bury et al., 1998).

Cell apoptosis has been widely studied in humans and other mammals systems (Tran et al., 2004), invertebrate models such as C. elegans (Lettre and Hengartner, 2006), and fish (Krumshnabel and Podrabsky, 2009). This programmed cell death removes unwanted or damaged cells in the course of development and differentiation, or in response to various forms of cellular damage (Taylor et al., 2008). In mammals, there are two major apoptotic pathways, which include an extrinsic pathway and an intrinsic, or mitochondrial, pathway. An extrinsic pathway is initiated by ligands, such as tumor necrosis factor (TNF-α), to form a ligand-receptor complex that activates caspase-8. This in turn activates caspase-3 to induce cell apoptosis (Tran et al., 2004). The intrinsic pathway can be stimulated by reactive oxygen species, intracellular stress, and intracellular damage to activate caspase-3 (Ferri and Kroemer, 2001; Hand and Menze, 2008). Caspase-3, involved in both apoptotic pathways, is a family of highly selective proteases that cleaves substrates using a cysteine residue (Ferri and Kroemer, 2001; Hand and Menze, 2008). Although differences exist in the process of apoptosis between fish and mammals, fish express virtually all of the core components of the mammalian
apoptotic machinery (Krumschnabel and Podrabsky, 2009). As the terminal event preceding cell death, caspase-3 elicits similar effects in both fish and mammals (Krumschnabel and Podrabsky, 2009; Kuida et al., 1996). Caspase-3, which was the first caspase identified in teleosts, plays a critical role in development by removing unwanted cells (Yabu et al., 2001), as well as a central role in the execution-phase of cell apoptosis induced by different stressors, such as hypoxia, bacterial infection, or hyperthermia in zebrafish (Lee et al., 2008), Atlantic salmon (Takle et al., 2006), sea bass (Reis et al., 2007), rainbow trout (Rojo and Gonzalez, 1999), and sturgeon (Lu et al., 2005).

1.4 Transcriptional and enzymatic activities of key enzymes

Although the capacity for gill remodeling to variable environmental salinity is well reported, few investigations have described the cellular mechanisms that regulate the transient and long-term responses of the organ to osmotic challenges. In a recent transcriptomics study, hypoosmotic shock was shown to alter the expression of 498 genes (out of approximately 6,800 genes) in the gills of F. heteroclitus during the time course of acclimation (Whitehead et al., 2011; Whitehead et al., 2012). These transcripts consisted of the early, transient expression of genes involved in signaling and crisis control and the altered expression of genes affecting cell morphology, osmoregulation, and energy metabolism. Transcripts for apoptotic genes, such as cytochrome c, caspase-8, cyclin-dependent kinase, and p38 MAP kinase, were upregulated upon hypoosmotic challenge (Whitehead et al., 2011; Whitehead et al., 2012). Additionally, arginase II (arg II) and ornithine decarboxylase (odc) mRNA levels were among the quickest and most significantly upregulated by freshwater transfer. These transcripts encode for two
enzymes that are involved in polyamine biosynthesis (Whitehead et al., 2011; Whitehead et al., 2012).

1.4.1 Distribution and function of arginase

Arginase is an enzyme containing a binuclear manganese ion that catalyzes the hydrolysis of L-arginine to form ornithine and urea (Di Costanzo et al., 2007). It has been observed in almost all organisms from bacteria (Soru and Zaharia, 1970) and plants (Muszynsk and Reifer, 1968) to invertebrates (Lisowskamyjak et al., 1978) and vertebrates (Yu et al., 2003). In most mammals, arginase exists in at least two isoforms, including arginase I (Arg I) and arginase II (Arg II) (Jenkinson et al., 1996; Spector et al., 1994). Although the three-dimensional crystalline structures of the two isoforms are nearly identical, their distribution and function differ (Cama et al., 2003). Arg I is primarily localized in hepatocytes, and it has been observed in many extrahepatic tissues such as salivary glands, esophagus, stomach, pancreas, thymus, leukocytes, skin, preputial gland, uterus, and sympathetic ganglia in mammals (Yu et al., 2003), where it functions mainly in the ornithine-urea cycle (OUC) (Jenkinson et al., 1996). Arg II is widely expressed in many extrahepatic tissues, such as intestine and kidney in mammals (Gotoh et al., 1996). In contrast to extrahepatic Arg I, extrahepatic Arg II has been implicated in the regulation of arginine and ornithine balance in the venular endothelia cells of bovine (Li et al., 2001). Arg II also appears to provide ornithine for the production of putrescine, the smallest of the major polyamines (Jenkinson et al., 1996).

The biological functions of arginase isoenzymes are different in ureotelic and ammoniotelic species. In ureotelic species, such as mammals, cytosolic arginase I converts arginine to ornithine, which is shuttled back into mitochondria for incorporation
into the OUC (Saha and Ratha, 1989; Jenkinson et al., 1996). In contrast, most water-breathing organisms, such as teleost fish and other aquatic invertebrates, are ammoniotelic, in that they lack a functional OUC and excrete their nitrogenous wastes as ammonium or ammonia (Hung et al., 2009). In ammoniotelic species, arginase II catalyzes the reaction converting arginine to ornithine, but do not shuttle this product into the OUC. Rather, ornithine is metabolized to proline, glutamate, aspartate, glutamine and polyamines (Gotoh et al., 1996).

Arginase is ubiquitous in different tissues among fish species (Portugal and Aksnes, 1983). Previous studies have shown that type I and type II arginase (similar tissue distribution to mammalian Arg I and Arg II) were observed in rainbow trout (Portugal and Aksnes, 1983; Wright et al., 2004). Moreover, the subcellular localization of arginase is related to its functions in fish. Mommsen and Walsh (1989) proposed that hepatic arginase I began to function in the OUC of the lungfish when its subcellular distribution became cytosolic rather than mitochondrial (Mommsen and Walsh, 1989). In contrast, coelacanths, marine elasmobranchs, and most teleosts all express Arg II within hepatic mitochondria. Although it serves no role in the OUC, Arg II plays an important role in regulating the synthesis of polyamines and proline (Mommsen and Walsh, 1989; Srivastava and Ratha, 2010). The availability of expressed sequence tag (EST) for killifish (F. heteroclitus) poses the idea that analogously mammalian Arg I and Arg II genes may exist in killifish (F. grandis).

1.4.2 Degradation of ornithine decarboxylase

Ornithine decarboxylase (Odc) is a pyridoxal 5’-phosphate-dependent enzyme that is a homodimer (Tobias and Kahana, 1993). Its active sites are formed at the dimer
interface between the NH$_2$-terminal domain of one subunit and the COOH-terminal domain of another subunit (Bercovich and Kahana, 1993). Odc has a very rapid turnover rate, with a half-life of less than one hour in brine shrimp and mammals (Svensson et al., 1997; Watts et al., 1996). One inhibitory protein of Odc is called antizyme, which works as a non-competitive inhibitor to bind monomer Odc COOH terminus. The binding of antizyme to the carboxyl terminus of Odc forms an inactive antizyme-Odc complex that inhibits enzyme activity (Hascilowicz et al., 2002) and exposes its C-terminus to proteolysis (Bercovich and Kahana, 1993; Coffino, 2001; Heller et al., 1976). Moreover, α-difluoromethylornithine (DMFO), an irreversible inhibitor of Odc, will inactivate the enzyme and deplete polyamine reserves upon binding to it (McCann and Pegg, 1992).

DFMO-induced polyamine depletion can impair intestinal epithelial growth (Bhattacharya et al., 2009), induce intestinal cell apoptosis in mice (Yerushalmi et al., 2006), and arrest human melanoma cells in the G1 phase (Kramer et al., 2001). At low concentrations, DFMO depleted intracellular polyamine stores and inhibited cell growth in human malignant glioma cells (Hunter et al., 1990). DFMO may alter a fundamental feature of cochlear spiral and cause a hearing loss. DFMO-induced hearing loss may be mediated through inhibition of polyamine synthesis in cochlea, consequently altering inward rectification of inner ear-specific Kir currents (Nie et al., 2005). The effects of DFMO in suppressing the outward component of Kir currents in other systems have been shown, the functional and clinic significance of DFMO is undetermined (Nichols and Lopatin, 1997). Moreover, the presence of a DFMO-insensitive decarboxylase was also detected in brine shrimp in response to altered salt concentrations, and the appearance of this enzyme may represent an alternative mechanism for polyamine synthesis (Watts et
Thus, DFMO is widely used for studying polyamine functions in biological and medical fields.

1.4.3 Distribution and function of polyamine oxidase

Polyamines are produced in part by the coordinated actions of Arg II and Odc. Polyamines are a diverse class of aliphatic molecules that are highly polycationic at physiological intracellular pH (Galston, 1983; Pegg and McCann, 1982; Sekowska et al., 1998). They have high reactivity to a diverse range of molecules, including DNA, RNA, and proteins, and perform a large number of physiological functions, such as RNA transcription, cell growth, cell proliferation, and apoptosis in animals and plants (Galston, 1983; O’Brien et al., 1975; Sekowska et al., 1998). Physiological levels of the polyamines putrescine, spermidine, and spermine are maintained in cells by both polyamine biosynthetic and catabolic pathways, as shown in Figure 1.2, and by regulating oncogenes to form a complex and regulated network, as shown in Figure 1.3.

The activities of Arg II, Odc, SSAT, and Pao contribute to the maintenance of polyamine concentrations at the physiological level in organisms (Pegg, 1988). Once Odc converts ornithine to putrescine, this molecule can be further aminated to spermidine and spermine via the activity of spermidine synthase and spermine synthase, respectively. Conversely, the polyamine catabolic pathway includes an acetylation reaction by spermidine/spermine N\(^1\)-acetyltransferase (SSAT) and the oxidation of N\(^1\)-acetylspermine by polyamine oxidase (Pao). Combining SSAT and Pao (SSAT/Pao) can convert spermine to spermidine, which, in turn, can be converted to putrescine (Figure 1.2). Although SSAT is a rate-limiting enzyme for polyamine catabolism, Pao is the terminal oxidase that regulates intracellular polyamine levels (Thomas and Thomas, 2001).
Figure 1.2: Flowchart of the major steps in the mammalian polyamine metabolic pathway (Wang et al. 2006). Note: 1-arginase II; 2-ornithine decarboxylase; 3-spermidine synthase; 4-spermidine synthase; 5-spermidine/spermine N\(^1\)-acetyltransferase; 6-polyamine oxidase. Steps 1-4 in blue refer to the biosynthetic pathway, in which ornithine is converted to putrescine by the action of ornithine decarboxylase. Through a series of amination reactions, putrescine can be converted to spermidine and spermine. Steps 5 and 6, shown in red, are part of polyamine catabolic pathway. Through a series of acetylation and oxidation reaction, spermine and spermidine are converted to spermidine and putrescine by the action of SSAT and polyamine oxidase.

Pao, a FAD-dependent oxidase that preferentially oxidizes N\(^1\)-acetylated polyamine products, was first purified in rat liver (Holtta, 1977). At least two isoforms of Pao exist in barley (Cervelli et al., 2006), and Pao activity has been observed in rat and human tissues such as liver, testis, kidney, spleen, small intestine, heart, brain, lung, and pancreas (Pavlov et al., 1991; Suzuki et al., 1984). Its wide tissue distribution suggests
Figure 1.3: The interactions among Myc, ornithine decarboxylase, and polyamines are shown. Myc genes respond to an extracellular signal and activate Odc translation, which produces the rate-limiting enzyme for polyamine biosynthesis. Polyamine levels have a positive feedback on Myc protein expression and form a positive loop. Myc protein directly participates in multiple physiological functions such as DNA stabilization and cell growth. α-difluoromethylornithine (DFMO), an inhibitor of Odc, inhibits Myc protein expression and reduces polyamine levels.

that the regulation of the relative levels of polyamines via modulation of polyamine catabolism is important in multiple tissues in mammals. Regardless, little is known of the distribution of Pao activity in teleost fish, nor how it is regulated during osmotic stress. Pao promotes apoptosis by damaging DNA or lipids (Campestre et al., 2011; Chaturvedi et al., 2004; Li et al., 2004; Wen et al., 2011), and regulates the ratio of spermine and spermidine (Adibhatla et al., 2002). Spermine can function as an antioxidant at high concentrations (Lovaas, 1995) or as a free radical scavenger (Ha et al., 1998) to benefit cell growth. Moreover, H₂O₂, which is a by-product of Pao, has controversial functions in organisms. It can act as an anti-bacterial (Derksen et al., 1999)
or fungicidal agent (Marking et al., 1994; Zhang et al., 2004) in fish. In contrast, H$_2$O$_2$ can induce cell apoptosis in mammals by multiple pathways (Chaturvedi et al., 2004; Demmano et al., 1996; Guan et al., 2011). Therefore, studying the activity of Pao and its by-product provides further understanding of the polyamine catabolism pathway.

1.5 Physiological function of polyamines to salinity challenge

Environmental challenges such as oscillations in salinity, dissolved oxygen, and temperature are known to influence polyamine levels in various organisms. Hyper-salinity exposure resulted in an increase of putrescine, spermidine, and spermine concentrations in plants (Campestre et al., 2011), in the gills of the blue crab, *C. danae* (Silva et al., 2008), and in the gills of *C. sapidus* (Lovett and Watts, 1995). Furthermore, hypoosmotic exposure in brine shrimp increased Odc activity and putrescine concentration, but had no effect on spermidine and spermine concentrations (Watts et al., 1996). The collective effect of increasing the mRNA levels of *arg II* and *odc* and reducing the concentration of intracellular polyamine binding proteins during hypoosmotic shock presumably increased free intracellular polyamine concentrations in *F. heteroclitus* (Whitehead et al., 2011), suggesting a possible role of polyamines in the short-term response to hypoosmotic challenges. Although the role of polyamines during freshwater challenges is unknown, upregulation of intracellular polyamines or the exogenous addition of polyamines causes apoptosis, which may be an important mechanism in gill remodeling (Laurent et al., 2006) or in regulation of cell volume. Therefore, it is plausible that there exists a close relationship between gill remodeling, Odc expression, and polyamine levels during hypoosmotic challenges.
1.6 Role of polyamines in cell proliferation and cell apoptosis

Polyamines appear to play a dual role in facilitating cell apoptosis and cell proliferation. Polyamines, the oxidized products of polyamines, and Odc can induce cell apoptosis, a biological process that is essential for physiological alterations in epithelia during osmotic challenges (Laurent et al., 2006). Spermine can activate caspases and initiate apoptosis (Stefanelli et al., 1998; Stefanelli et al., 1999). Moreover, polyamine-oxidized products, such as reactive oxygen species (ROS) and polyamine oxidase, promotes apoptosis by damaging DNA or lipids (Campestre et al., 2011; Chaturvedi et al., 2004; Li et al., 2004; Wen et al., 2011). Upregulation of spermine oxidase can initiate apoptosis by oxidative stress or depolarization of mitochondrial membranes (Chaturvedi et al., 2004). Ornithine decarboxylase can induce apoptosis by triggering the c-Myc pathway (Packham and Cleveland, 1995; Packham et al., 1996) or can induce spermidine accumulation to trigger apoptosis (Poulin et al., 1995; Poulin et al., 1989). Inhibition of ornithine decarboxylase activity with DFMO can inhibit cell apoptosis by depletion of polyamines (Deng et al., 2005; Monti et al., 1998).

Polyamines are also involved in diverse physiological functions, including the regulation of cell proliferation and apoptosis. Polyamines are involved not only in normal cell proliferation and differentiation, but also in malignant transformation. They can positively regulate proto-oncogenes, such as c-fos, c-ras, and c-myc transcription (Bachrach et al., 2001; Li et al., 1998; Liu et al., 2005; Tabib and Bachrach, 1994; Tabib and Bachrach, 1999; Thomas and Thomas, 2003). DFMO application can induce polyamine depletion and reduce c-myc and c-fos mRNA levels in cells (Celano et al., 1988; Tabib and Bachrach, 1994; Wang et al., 1993). These findings suggest that high
levels of polyamines are associated with many human cancers (Thomas and Thomas, 2003). In addition, polyamines can negatively regulate the expression of growth-inhibiting genes, such as p53, and stimulate cell growth (Bhattacharya et al., 2009). DFMO has been shown to reduce polyamine levels in intestinal epithelia cells by inhibiting c-myc and c-fos expression (Li et al., 2000) and upregulating p53 protein expression to hinder cell growth (Bhattacharya et al., 2009; Ray et al., 1999).

Proto-oncogenes increase protein levels, such as c-Myc and c-Fos, via extracellular stimuli, such as selenium (Yu et al., 2006), oxidative stress (Ichiki et al., 2003), and salinity (Herbert, 1996); consequently, Odc activity and polyamine concentrations were increased (Figure 1.3) (Packham et al., 1996). These findings suggest that a positive feedback loop exists among Myc and/or Fos proteins, Odc, and polyamines, and that these exhibit physiological impacts as shown in Figure 1.3 (Packham et al., 1996). Although increased Odc activity promotes polyamine biosynthesis, polyamines have an inhibitory effect on odc mRNA expression (Lovkvist Wallstrom et al., 2001). Therefore, a complex interplay exists in the cells in order to support numerous physiological functions of polyamines in animals and plants (Galston, 1983; O'Brien et al., 1975; Sekowska et al., 1998).

1.7 Brief outline of main points of each chapter

Despite recent advances, little is known of the role of polyamines in F. grandis when faced with hypoosmotic or hyperosmotic challenges. In this dissertation, an integrative approach, spanning cellular and molecular biology, was utilized to study the role of polyamines in the short-term and compensatory responses of adult F. grandis to hypoosmotic challenges.
Chapter 2: This chapter aimed to assess the mRNA levels and enzymatic activities of Arg II and Odc and polyamine concentrations during hypoosmotic challenges. Overall, my results demonstrated that arg II and odc mRNA levels were highly upregulated in the gills, with an inverse relationship of salinity on Odc increase during the first few days post-transfer to hypoosmotic conditions. DFMO inhibited polyamine biosynthesis and Odc activity following hypoosmotic exposure. Furthermore, polyamine biosynthesis mediated the early-phase compensatory response of hypoosmotic shock in the euryhaline Gulf killifish.

Chapter 3: This study aimed to investigate the role of polyamines in gill epithelial remodeling and opercular epithelial cell swelling. The opercular epithelium is a surrogate model of the marine fish gill that has been used to study the molecular events associated with cell swelling and cell volume regulation. Scanning electron microscopy showed that exposure to fresh water (0.1 ppt) promoted a morphological shift in the MRCs of the gill epithelium from a SW-type to FW-type. Moreover, addition of exogenous polyamines, especially spermidine, to the basolateral side of opercular epithelium during hypotonic exposure, inhibited the decrease of short-circuit current (Isc) and membrane conductance, suggesting opercular cells may swell by inhibition of Cl− secretion. The putative roles of polyamines in regulating cell volume need to be further studied.

Chapter 4: This study aimed to assess the tissue distribution of key enzymes for polyamine metabolism. This study demonstrated that hypoosmotic challenges increased the transcription and enzymatic activities of arginase, ornithine decarboxylase, and polyamine oxidase in the gills, intestine, and liver. Polyamine metabolism was
upregulated in multiple tissues, suggesting that polyamine levels were regulated in the whole body of the killifish.
CHAPTER 2: THE INFLUENCE OF HYPOOSMOTIC EXPOSURE ON POLYAMINE BIOSYNTHESIS IN THE GULF KILLIFISH

2.1 Introduction

Polyamines are a diverse class of aliphatic, highly reactive molecules due to their polycationic chemistries at physiological intracellular pH (Galston, 1983; Sekowska et al., 1998). As such, they have an astonishing array of physiological functions in microbes, animals, and plants (Galston, 1983; O'Brien et al., 1975; Sekowska et al., 1998), including their ability to stabilize DNA, RNA, and phospholipids regulate ion channel activity, facilitate tissue remodeling, and aid in cell growth and differentiation (Pegg and McCann, 1982; Ray et al., 1999; Tabor and Tabor, 1984). Impairment of this homeostatic balance can produce severe biological consequences, including nervous system injury in rat (Adibhatla et al., 2002) and tumor growth in mammal (Thomas and Thomas, 2003). Due to their reactivity, strict control of polyamine concentrations is necessary for their physiological functions.

Polyamines are synthesized in part by the coordinated actions of arginase II (Arg II) and ornithine decarboxylase (Odc). Ornithine is the major precursor of polyamines and is synthesized from arginine by mitochondrial Arg II. Once produced, ornithine decarboxylase can convert ornithine to putrescine in an irreversible and rate-limiting reaction (Pegg and McCann, 1982). Putrescine, which is a diamine, can be converted to spermidine and spermine, which are triamine and tetramine compounds, respectively. Once formed, spermidine and spermine can be catabolized back to putrescine in order to maintain specific ratios of polyamines (Adibhatla et al., 2002). Although under tight control, the concentrations of these three polyamines are highly affected by various physiological and environmental stimuli, including osmotic challenges (Zapata et al.,
2004), ozone (Langebartels et al., 1991), and UV radiation (Zacchini and de Agazio, 2004) in plants. A recent transcriptomic study on the impacts of hypoosmotic shock in the euryhaline teleost fish, *F. heteroclitus*, showed that gill odc mRNA is highly upregulated almost immediately following acute exposure to fresh water (Whitehead et al., 2012). It was also observed that the concentration of a gill mRNA expressing a polyamine-binding protein was decreased to non-detectable levels following freshwater exposure (0.1 ppt) in *F. heteroclitus* (Whitehead et al., 2012). Another study observed that the mRNA level of antizyme, a key inhibitor of Odc activity *in vivo*, was decreased in response to hypoosmotic exposure (Mitchell et al., 1998). The collective effect of increasing polyamine biosynthesis and reducing intracellular polyamine binding would presumably increase free intracellular polyamine levels. In comparison, the striped killifish *F. majalis*, which is unable to tolerate freshwater, showed little upregulation in polyamine biosynthetic transcripts during hypoosmotic exposure (Whitehead et al., 2013). These data support a possible role of polyamines in either the short-term or the compensatory response of these euryhaline fish to hypoosmotic challenges.

The main goal of this study was to assess the influence of acute hypoosmotic challenges on the mRNA levels and enzymatic activities of Arg II and Odc in the gills of the Gulf killfish, *F. grandis* (Hsiao and Meier, 1989). Both *Fundulus* species are accepted models for studying the physiological basis of environmental challenges (Burnett et al., 2007) because they have abilities to tolerate frequent and episodic oscillations in environmental salinity, dissolved oxygen, and temperature (Marshall, 2003; Wood and LeMoigne, 1991). The gill was investigated because it is one of the primary organs involved in fish osmoregulation (Evans et al., 2005). This study also
investigated the effects of alpha-DL-difluoromethylornithine (DFMO), an irreversible inhibitor of Odc (McCann and Pegg, 1992), on polyamine regulation during hypoosmotic challenges in the gill of *F. grandis*.

**2.2 Materials and methods**

**2.2.1 Experimental animals**

Adult *F. grandis* were obtained from a local hatchery (Gulf Coast Minnows, Thibodeaux, LA, USA) and held in a 570-L recirculation system maintained in the Life Sciences Aquatic Facility at Louisiana State University (Baton Rouge, LA, USA). Four weeks prior to experimentation, fish were randomly transferred to five 110-L aquaria plumbed on a single recirculation system with mechanical and biological filtration and UV-sterilization. Water salinity was adjusted by diluting sea salt (Instant Ocean®, Blacksburg, VA, USA) with reverse osmosis water, and water salinity and temperature (23 ± 1 °C) were monitored daily using a water quality meter (YSI, Yellow Spring, OH, USA). Partial replacement of system water was performed at least twice per week to keep ammonia and nitrite below the detection levels of commercial kits (Aquarium pharmaceuticals, Chalfont, PA, USA), and dissolved oxygen was always near air-saturation using a low-pressure aerator. A 12 h light:12 h dark cycle was provided by fluorescent lighting. Fish were fed a commercial pellet (Cargill, Franklin, LA, USA) twice daily at 2% body weight per day throughout the study.

**2.2.2 Experimental design**

In the first series of experiments, one hundred and fifty adult fish (weight range of 4.3-16.8 g) were acclimated to 5 ppt water for at least one month and fed twice daily, except for 24 h before salinity transfer. Fish were sampled immediately prior to transfer
or at 6 h, 1 d, 3 d, or 7 d post-transfer to 5, 2, 1, 0.5, or 0.1 ppt water (N = 6 per time point per salinity). Fish were net-captured and anesthetized in 0.5 g/L tricaine methanesulfonate (MS-222). Whole gill baskets were dissected from fish and washed with ultrapure water for 10 s. The first two left gill arches were immersed in RNAlater and stored at 4 °C overnight, and transferred to -20 °C. Total RNA extraction and quantitative PCR (qPCR) analysis of argII and odc transcript abundance were performed as described in Section 2.2.3. All four gill arches from the right side were flash frozen in liquid nitrogen and stored at -80 °C. The 1st and 2nd right gill arches were analyzed for Arg activity, and the 3rd and 4th gill arches were analyzed for Odc activity, as described in Sections 2.2.4 and 2.2.5, respectively.

In a second series of experiments, the effect of salinity on gill polyamine concentrations was assessed. Sixty adult fish (weight range of 4.28 to 11.35 g) were acclimated to 5 ppt water for at least one month, then were sampled randomly prior to transfer or at 6 h, 1 d, 3 d, or 7 d post-transfer to 5 ppt or 0.1 ppt water (N = 6 per time point per salinity). Whole gill baskets were dissected from fish, washed with ultrapure water, flash frozen in liquid nitrogen, and then stored at -80 °C while awaiting analysis of polyamine concentrations as described in Section 2.2.6.

In addition, the influence of ornithine decarboxylase inhibition on gill polyamine content in fish undergoing salinity challenges was assessed. Two hundred and forty adult F. grandis (weight range of 4.9-12.3 g) were acclimated to 5 ppt water. From two days prior to the start of salinity transfer to one week post-salinity transfer, fish were injected intraperitoneally once daily at 10 µL/ g with: (i) phosphate-buffered saline (PBS; sham controls) consisting of 146 mM NaCl, 3 mM KCl, 15 mM NaH₂PO₄, 15 mM Na₂HPO₄,
10 mM NaHCO₃ (pH 7.4), or (ii) 0.2 mg/µL of alpha-DL-difluoromethylornithine (DFMO) dissolved in PBS. The dosage of DFMO that affected Odc activity without fish mortality was determined by pre-experiment (data not shown). Whole gill baskets prior to salinity transfer (N=6) were sampled. On the third day of injections, half of the PBS-injected fish (sham control) and half of the DFMO-injected fish were transferred to 0.1 ppt water, whereas the remaining PBS and DFMO injected fish were maintained at 5 ppt; daily injections continued for a week post-transfer. In total, fish were allotted to one of four experimental treatments, including: i) 5 ppt with PBS, ii) 5 ppt with DFMO, iii) 0.1 ppt with PBS, or iv) 0.1 ppt with DFMO. Whole gill baskets were sampled at 6 h, 1 d, 3 d, and 7 d post transfer (N=6 per time point per salinity) and stored at -80 °C before analyzing Odc activity (Section 2.2.5), and polyamine concentrations (Section 2.2.6).

2.2.3 arg II and odc mRNA levels

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for the extraction of total RNA according to the manufacturer’s instructions. Total RNA concentrations were determined by measuring the absorbance at 260 nm and 280 nm with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The ratio of absorbance at 260 nm to 280 nm was used to assess the purity of RNA, with a ratio of 1.9 to 2.1 used as the criterion for optimal RNA quality. Single-stranded cDNA was synthesized from 2 µg total RNA using random primers with a commercial reverse transcription kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Carlsbad, CA, USA).

Expressed sequence tags (EST’s) for arg II (AB290198.1) and odc (CN977303.1) from F. heteroclitus were obtained from the National Center for Biotechnology Information, NCBI.
Information database (Bethesda, MD, USA). Primers for use in quantitative PCR were developed for both genes using Integrated DNA Technologies Inc. (Coralville, IA, USA) software (Table 2.1).

Table 2.1. Sequences of forward and reverse primers used for amplification of *F. grandis arg II*, *odc*, and *18s rRNA* amplicons for quantitative RT-PCR analysis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Direction</th>
<th>Sequence 5’→3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>argII-qPCR</td>
<td>Forward</td>
<td>CAGCTGTGGTTACTGCGATTGCGTT</td>
</tr>
<tr>
<td>argII-qPCR</td>
<td>Reverse</td>
<td>ACAGGAAGCAACAAAAACCAGCACAG</td>
</tr>
<tr>
<td>odc-qPCR</td>
<td>Forward</td>
<td>TTGCCACGCCAATGTGCTTCAA</td>
</tr>
<tr>
<td>odc-qPCR</td>
<td>Reverse</td>
<td>ATGCTCCTGTCAAGTAACTGGCCT</td>
</tr>
<tr>
<td>18s rRNA-qPCR</td>
<td>Forward</td>
<td>TTCCGATAACGAACGAGAC</td>
</tr>
<tr>
<td>18s rRNA-qPCR</td>
<td>Reverse</td>
<td>GACATCTAAGGGGCATCACAG</td>
</tr>
</tbody>
</table>

qPCR analysis was performed for the target genes, *arg II* and *odc*, and the reference gene, 18S ribosomal RNA (*18s rRNA*), using a commercial qPCR kit (SYBR Green core Reagent, Applied Biosystems, Carlsbad, CA, USA) with an ABI PRISM 7000 Sequence Detection System (Carlsbad, CA, USA). Primer pairs amplified single amplicons as demonstrated by 2% agarose gel electrophoresis and by denaturation analysis following individual qPCR runs. Reactions were performed with 0.1 µg first-strand cDNA, 10 nM forward and reverse primers, and 5 µL SYBR Green reagents in a total volume of 20 µL. Samples underwent 40 PCR cycles at 95 °C for 15 s and 60 °C for 1 min. Adaptive baselines were used for background detection. The critical threshold (Ct), the cycle number at which cDNA amplification entered an exponential increase, was
calculated automatically using preset algorithms within the software. Preliminary experiments were performed to calculate qPCR efficiencies for each gene product at varying concentrations of 1st strand cDNA over a concentration range spanning two orders of magnitude. One randomly selected control sample (5 ppt pre-transfer control) was used to develop a standard curve relating Ct to the relative quantity of cDNA. From this curve, efficiency (E) was calculated from the linear regression of Ct versus log cDNA for each gene of interest according to the formula:

\[
E = [10^{(1/slope)}] - 1 \tag{Equation 2.1}
\]

Relative change (R) in mRNA levels were measured using the \(2^{-\Delta Ct}\) method (Fleige et al., 2006) following the efficiency correction as described by Pfaffl (2001) and is summarized in Equation 2.2:

\[
R = \frac{(E_{target})\Delta Ct_{target} (control–treatment)}{(E_{ref})\Delta Ct_{ref} (control–treatment)} \tag{Equation 2.2}
\]

Target refers to either arg II or odc, and ref refers to the reference gene 18s rRNA. Efficiency (E) was calculated for each primer set according to Equation 2.1. Reaction efficiencies for the target and reference genes were between 90 to 110%, with 100% efficiency representing a doubling in the amount of cDNA per cycle. \(\Delta Ct\) represents the difference in Ct between the target genes (arg II or odc) and the reference gene, 18s rRNA.

2.2.4 Gill total Arg activity

Total Arg activity was measured from the rate of urea production resulting from the enzymatic conversion of arginine to ornithine as described by Mommsen (1983). Gill arches were thawed and immediately homogenized in ice-cold 50 mM HEPES buffer (V:V for 1:5) using a PRO200 Homogenizer (Lab Depot Inc. Dawsonville, GA, USA).
Homogenates were centrifuged at 12,000 g for 4 min at 4 °C, then the decanted supernatants were pre-incubated with 50 mM MnCl₂ for 10 min for maximum activation of the enzyme (Mommsen et al., 1983). Assays were initiated by the addition of 10 µL of supernatant, 10 µL 50 mM MnCl₂, and 480 µL reagent mix, consisting of 50 mM HEPES (pH 8.0), 250 mM L-arginine, and 1 mM MnCl₂. Assays were conducted at room temperature (23-25 °C). Reactions were terminated after 30 min by the addition of 25 µL 70% perchloric acid, then neutralized with KHCO₃. Each reaction mix was centrifuged at 12,000 g for 4 min at 4 °C, and the resulting supernatant was analyzed for urea according to the QuantiChrom urea assay kit instructions (Bioassay System, Hayward, CA, USA). Total Arg activity was expressed in µmol urea produced /h/g gill.

2.2.5 Gill ornithine decarboxylase activity

Preliminary tests indicated no significant difference in ornithine decarboxylase enzymatic activity between samples, whether they were flash frozen in liquid nitrogen and stored at -80 °C before analyses or if analyzed fresh following tissue sampling (data not shown). Consequently, frozen gill samples were thawed on ice, homogenized in reaction buffer (50 mM K₂HPO₄, 5 mM dithiothreitol, 0.2 mM EDTA, 0.05 mM pyridoxal 5-phosphate, pH 7.5), and centrifuged at 20,000 g for 20 min at 0 °C. The supernatant was decanted into a 16 mm x 100 mm borosilicate tube sealed with a double seal rubber stopper (Kimble Chase Kontes, K-882310) and penetrated by a plastic centerwell (Kimble Chase Kontes, K-882320). The centerwell contained a 1.3 cm² of Whatman #1 filter paper saturated with 133 µL 40% KOH. At the start of the assay, a 0.5 µCi mixture of DL-[1-14C] ornithine hydrochloride (52 mCi/mmol, Moravek Biochemicals, Brea, CA, USA) and cold L-ornithine (Sigma-Aldrich, St. Louis, MO,
USA) were added to the air-tight borosilicate tube to reach a final ornithine concentration of 1.2 mM, as described by Watts (Watts et al., 1996). The reaction mixture was kept in a water bath at 25 °C for 90 min before being terminated with the addition of 5% trichloroacetic acid. $^{14}$CO$_2$ produced by the enzymatic reaction was collected overnight on filter paper saturated with 40% KOH. Filter paper was placed into 5 mL Ultima Gold cocktail (Perkin-Elmer, Waltham, MS, USA), then counted for radioactivity using a liquid scintillation analyzer (Tri-Carb 2900TR, Perkin-Elmer, Waltham, MS, USA), as described by Watts (Watts et al., 1996). Internal standardization demonstrated both constant and negligible quench, so no correction of counting efficiency was necessary. Odc activity was expressed as nmol CO$_2$ produced /h/g gill.

2.2.6 Gill polyamine contents

Gill baskets were thawed and homogenized with methanol (containing 0.1 mM HCl) (V:V for 1:3) in dry ice using a PRO200 Homogenizer (Lab depot Inc. Dawsonville, GA, USA). Samples were incubated in 0.6 M perchloric acid to extract polyamines and derivatized with o-toluoyl chloride using a slightly modified procedure from that described previously (Wongyai et al., 1988). Samples (20 µL) were then analyzed for polyamines by high performance liquid chromatography (HPLC) (Bio-Rad high-performance liquid chromatograph gradient module with a Model 996 photodiode array detector; Milford, MS, USA). HPLC separations were performed using an ACE C-18-PFP column (150 mm x 4.6 mm; 5 µm; Chadds Ford, PA, USA), a mobile phase consisting of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile, with a flow rate of 1.0 mL/min, and UV detection at 254 nm.
2.2.7 Polyamine levels during inhibition of Odc activity in gills

Gill Odc activity and polyamine concentrations of DFMO-injected and PBS-injected fish at 6 h to 7 d post-transfer to 5 ppt or 0.1 ppt water were measured in this study. The percent change in gill Odc activity and polyamine concentrations of DFMO-injected and PBS-injected fish relative to the simultaneous controls at 6 h to 7 d post-transfer to 5 ppt or 0.1 ppt water, as described in Section 2.2.5, was calculated as shown in Equation 2.3,

\[
\% \text{ change} = \frac{\text{Value}_{\text{post-transfer}} - \text{Value}_{\text{sham control}}}{\text{Value}_{\text{sham control}}} \times 100
\]  
(Equation 2.3)

where value\_sham\_control represents gill Odc activities and polyamine concentrations measured at 6 h through 7 d post-transfer to 5 ppt or 0.1 ppt water.

2.2.8 Statistical analysis

Statistical differences in gill arg II and odc mRNA levels, enzymatic activity, and polyamine concentrations between treatments were analyzed using a one-way ANOVA with a Tukey post hoc test (v.9.3, SAS Institute Inc., Cary, NC, USA). Statistical differences between treatments, salinity and exposure time were determined using a two-way ANOVA. Statistical differences were considered significant at a P-value of 0.05 or less.

2.3 Results

2.3.1 Gill arg II and odc mRNA levels

Quantitative PCR analyses of arg II and odc mRNA levels were conducted to assess the influence of hypoosmotic exposure on the mRNA levels of transcripts encoding key enzymes for polyamine biosynthesis. The level of arg II mRNA in the gills
was inversely correlated with the salinity shortly following acute post-transfer to hypoosmotic conditions, tending to peak at 1d post-transfer, then gradually decreasing by 7 d post transfer in all hypoosmotic exposures (Figure 2.1 panel A). Gill arg II mRNA levels were highest in the 0.5 ppt and 0.1 ppt-acclimated fish between 6 h and 1 d following transfer relative to the pre-transfer controls ($P < 0.0001$ and $P < 0.0001$, respectively) (Figure 2.1 panel A). Gill arg II mRNA in the 0.1 ppt-acclimated fish were increased 10.5-fold ($P < 0.0001$) by 1 d post-transfer and remained elevated 2.7-times above pre-transfer controls by 7 d (Figure 2.1 panel A). Similarly, gill odc mRNA levels were strongly influenced by the magnitude of hypoosmotic challenges (Figure 2.1 panel B). Overall, there was a strong dose-dependent decrease in gill odc mRNA levels with decreasing salinity between 6 h to 7 d (Figure 2.1 panel B). Time and salinity were the main variables that altered odc mRNA expression ($P < 0.0001$ and $P < 0.0001$, respectively), and there was a statistical interaction between salinity and time ($P = 0.0002$) (Figure 2.1 panel B). The gill odc mRNA level increased approximately 21-fold at 6 h ($P = 0.002$) and 34-fold at 1 d ($P = 0.001$) post-transfer to 0.1 ppt water, but was still elevated approximately 4.5-fold above the level measured in gills of the 5 ppt control fish by 7 d post-transfer to 0.1 ppt water.

2.3.2 Gill total arginase and Odc activity

Because arg II (Figure 2.1 panel A) and odc mRNA levels were significantly upregulated (Figure 2.1 panel B) during hypoosmotic exposure, the influence of salinity on total Arg and Odc activities was investigated. Overall, the magnitude of total Arg activity was inversely proportional to the magnitude of hypoosmotic shock at 1 d and 7 d post-transfer (Figure 2.2 panel A) and was only increased by a maximum of
Figure 2.1: Relative mRNA levels of *arg II* (A) and *odc* (B) in gills of *F. grandis* acclimated to 5 ppt and sampled pre-transfer or at 6 h, 1 d, 3 d, and 7 d following acute transfer to 0.1, 0.5, 1, 2, 5 ppt as examined by quantitative RT-PCR analysis. Data are expressed as mean values ± SEM (N = 6) relative to 18s rRNA after normalizing each value to the *arg II* and *odc* mRNA level of gills from 5 ppt-acclimated fish pre-transfer. Asterisks indicate a significant difference (*P* < 0.05) in comparison to the 5 ppt treatment for that time point; different letters indicate a significant difference (*P* < 0.05) in comparison to the pre-treatment for different time points.
Figure 2.2: Total Arg activity (A) and Odc activity (B) in gills of *F. grandis* acclimated to 5 ppt water and sampled prior to transfer, or at 6 h, 1 d, 3 d, and 7 d following acute transfer to 0.1, 0.5, 1, 2, and 5 ppt water. Data are expressed as mean values ± SEM (N = 6). Asterisks indicate a significant difference (*P* < 0.05) in comparison to the 5 ppt treatment; different letters indicate a significant difference (*P* < 0.05) in comparison to the pre-treatment.
approximately 25% from the pre-transfer value at any given time during exposure (Figure 2.2 panel A). In comparison, gill Odc enzymatic activity increased almost in direct proportion to the reduction in water salinity at all time points except at 1 d post-transfer (Figure 2.2 panel B). Time and salinity were the two main variables that affected Odc enzymatic activity ($P < 0.0001$ and $P < 0.0001$, respectively), but there was no statistical interaction between them (Figure 2.2 panel B). Gill Odc activity was increased approximately 9.5-fold at 6 h ($P < 0.001$) and 4.9-fold at 7 d in 0.1 ppt-exposed fish ($P < 0.001$) compared to 5 ppt-acclimated fish (Figure 2.2 panel B). Odc activity in gills of 0.5 ppt-treated fish were also significantly elevated on 3 d and 7 d post-transfer compared to the Odc activity of 5 ppt-acclimated fish (Figure 2.2 panel B).

2.3.3. Gill polyamine contents

Based on the large stimulation of the polyamine biosynthetic pathway during hypoosmotic exposure (0.1 ppt), gill polyamine concentrations were measured. In accordance with the salinity-dependent increase in gill odc mRNA and Odc activity, the concentrations of putrescine, spermidine, and spermine in killifish gills were increased significantly by 0.1 ppt water exposure (Figure 2.3). Time and salinity were the two main variables to affect gill polyamine levels ($P<0.0001$ for both effects). Compared to pre-transfer levels, gill polyamine concentrations did not vary over time in the 5 ppt treatment; however, gill putrescine level increased after 1 d ($P<0.0001$) and remained elevated during the first 7 d post-transfer to fresh water (Figure 2.3 panel A). In comparison, gill spermidine (Figure 2.3 panel B) and spermine concentrations (Figure 2.3 panel C) were only transiently elevated, decreasing to pre-transfer levels after 3 d of freshwater exposure.
Figure 2.3: Putrescine (A), spermidine (B), and spermine (C) concentrations in gills of *F. grandis* acclimated to 5 ppt water then measured pre-transfer, or at 6 h, 1 d, 3 d, and 7 d post-transfer to 0.1 ppt or 5 ppt water. Data represented as mean values ± SEM (N = 6). In comparison to the level of 5 ppt treated fish, asterisks indicate a significant difference in the values of 0.1 ppt treated fish at the same time point (*P* < 0.05). In comparison to the pre-transfer level, different letters indicate a significant difference in the values of post-transfer fish to either 5 ppt or 0.1 ppt at different time points (*P* < 0.05).
The ratios of putrescine level to the sum of concentrations of spermidine and spermine were summarized in Table 2.2. Hypoosmotic exposure decreased this ratio at 6 h due to the transient accumulation of spermidine and spermine in the gill after acute salinity challenge (Table 2.2). Over time, spermine and spermidine concentrations dropped back to 5 ppt levels, whereas gill putrescine concentration remained elevated. The cumulative effect of these changes in polyamine concentrations over time explain why polyamine ratios (Table 2.2) increased significantly at 1 d and beyond.

<table>
<thead>
<tr>
<th></th>
<th>5 ppt</th>
<th>0.1 ppt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-transfer</td>
<td>0.86±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>----</td>
</tr>
<tr>
<td>6 h</td>
<td>0.94±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.81±0.04&lt;sup&gt;a,*&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 d</td>
<td>0.81±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.99±0.11&lt;sup&gt;a,*&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 d</td>
<td>0.82±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.12±0.16&lt;sup&gt;b,*&lt;/sup&gt;</td>
</tr>
<tr>
<td>7 d</td>
<td>0.88±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.31±0.06&lt;sup&gt;b,*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

In comparison to the level of 5 ppt treated fish, asterisks indicate a significant difference in the values of 0.1 ppt treated fish at the same time point (<i>P</i><0.01). In comparison to the pre-transfer level, different lower-case letters indicate a significant difference in the values of post-transfer fish exposed to either 5 ppt or 0.1 ppt at different time points (<i>P</i><0.05).

2.3.4 Pharmacological inhibition of ornithine decarboxylase activity

The effects of pharmacological inhibition of Odc activity on polyamine levels in the gill (Figure 2.4) during hypoosmotic challenge (0.1 ppt) were assessed. Gill Odc activity was not measured <i>in vivo</i>, but rather the maximal activity that presumably reflected the amount of active enzyme in the gill. When acclimated to 5 ppt water, gill Odc activity did not differ between the PBS and DFMO treatments, nor did it vary from
the pre-transfer level (Figure 2.4). In contrast, exposure to 0.1 ppt water increased gill Odc activity significantly at 6 h (39 %) and 1 d (33%) in the sham control fish (Figure 2.4). Following DFMO treatment, Odc activity was reduced by 21% at 1 d and 27% at 3 d significantly compared to the sham control undergoing hypoosmotic transfer (Table 2.3).

![Figure 2.4: Ornithine decarboxylase activity in gills of F. grandis exposed to 5 ppt and 0.1 ppt water for 7 d and given intraperitoneal injections of PBS or DFMO daily. The vertical dashed line divides pre-transfer and post-transfer measurements. Data are presented as mean value ± SEM (N = 6). Asterisks indicate a significant difference (P<0.05) in comparison to the PBS (sham control) fish; lower-case letters indicate a significant difference (P<0.05) in comparison to the pre-treated fish.](image)

The concentrations of putrescine, spermidine, and spermine in the gills of 5 ppt-acclimated fish did not vary over 7 d of sham injection (Figure 2.5). In comparison, gill polyamine concentrations in fish exposed to 5 ppt water, but injected with DFMO, decreased significantly (Table 2.3). Putrescine, spermidine, and spermine concentrations
were reduced by 86%, 62%, and 47% at 7 d post-transfer to 0.1 ppt water, respectively (Table 2.3).

Table 2.3. The percent change in gill Odc activity and polyamine concentrations of DFMO-injected and PBS-injected fish relative to the simultaneous controls at 6 h to 7 d post-transfer to 5 ppt or 0.1 ppt water.

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Odc</th>
<th>PUT</th>
<th>SPD</th>
<th>SP</th>
<th>Odc</th>
<th>PUT</th>
<th>SPD</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 h</td>
<td>-15%</td>
<td>-37%*</td>
<td>-26%*</td>
<td>-37%*</td>
<td>1%</td>
<td>-36%*</td>
<td>-28%*</td>
<td>-18%*</td>
</tr>
<tr>
<td>1 d</td>
<td>-21%*</td>
<td>-47%*</td>
<td>-52%*</td>
<td>-47%*</td>
<td>12%</td>
<td>-51%*</td>
<td>-29%*</td>
<td>-21%*</td>
</tr>
<tr>
<td>3 d</td>
<td>-27%*</td>
<td>-67%*</td>
<td>-24%*</td>
<td>-77%*</td>
<td>17%</td>
<td>-54%*</td>
<td>-24%*</td>
<td>-21%*</td>
</tr>
<tr>
<td>7 d</td>
<td>-7%</td>
<td>-75%*</td>
<td>-33%*</td>
<td>-65%*</td>
<td>10%</td>
<td>-86%*</td>
<td>-62%*</td>
<td>-47%*</td>
</tr>
</tbody>
</table>

Asterisks indicate a significant difference ($P<0.05$) in DFMO-treated fish in comparison to PBS-treated fish at any given time and salinity. All calculations were calculated using equation 2.3. Abbreviations: Odc, ornithine decarboxylase; PUT, putrescine; SPD, spermidine; SP, spermine.

Compared to pre-transfer levels, the putrescine level in the sham control group increased over time after transfer to 0.1 ppt water (Figure 2.5 panel A). Furthermore, DFMO inhibited the increase in putrescine associated with 0.1 ppt exposure at all time points (Table 2.3). Moreover, the spermidine concentration in gills of 5 ppt-acclimated fish was significantly reduced over time compared to those of the pre-transfer controls (Figure 2.5 panel B). Gill spermidine concentration after 1 d of 0.1 ppt exposure was increased, but returned to the pre-transfer level after 3 d (Figure 2.5 panel B). DFMO application decreased the spermidine level inducible by hypoosmotic exposure at 1 d and beyond (Table 2.3). Compared to the pre-transfer level, gill spermine concentration was only reduced at 7 d in 5 ppt-acclimated fish by DFMO application (Figure 2.5 panel C). Hypoosmotic exposure increased the spermine level at 1 d, yet had no significant effect.
after 3 d compared to the pre-transfer levels. (Figure 2.5 panel C). DFMO application decreased the spermine level inducible by hypoosmotic exposure at 3 d and beyond (Table 2.3).

Figure 2.5: Gill putrescine, spermidine, and spermine concentrations (N=6) in *F. grandis* acclimated to 5 ppt and 0.1 ppt water with PBS or DFMO treatment. Data are presented as mean value ± SEM (N = 6). Lower-case letters indicate a significant difference (P<0.05) in a pair-wise comparison.
2.4 Discussion

2.4.1 Increase in *arg II* and *odc* mRNA levels after hypoosmotic exposure

This study suggests that changes in the expression of genes associated with polyamine biosynthesis may be an important transcriptional response of euryhaline fish to acute hypoosmotic exposure. This study expands on this knowledge by describing the magnitude and timing of stimulation of polyamine synthesis, associating it with the early stages of phenotypic transition of the fish gill to fresh water (data not shown). The present study suggests that mRNA transcripts of *arg II* and *odc* were strongly expressed in the gills shortly after acute transfer to fresh water (0.1 ppt) and that gill mRNA levels were inversely correlated to environmental salinity (Figure 2.1 panel A and B). Moreover, enzymatic activity of Odc was upregulated following acute hypoosmotic exposure (Figure 2.2 panel B), suggesting a link between hypoosmotic exposure and Odc activity. A similar relationship between hypoosmotic challenge and Odc activity was described in the gills of *F. heteroclitus* (Whitehead *et al.*, 2012) and brine shrimp (Watts *et al.*, 1996), and in the variant L1210 mouse leukemia cell line (Poulin *et al.*, 1995). As a key enzyme for polyamine biosynthesis, Odc upregulation suggested a link between polyamine biosynthesis and hypoosmotic exposure. Although little is known of their function in fish, polyamines have important roles in cell proliferation and apoptosis (Pegg and McCann, 1982; Ray *et al.*, 1999; Tabor and Tabor, 1984). Cell proliferation and apoptosis contributed to the morphological modification in *F. heteroclitus* after hypoosmotic transfer (Katoh and Kaneko, 2003; Laurent *et al.*, 2006). There is a possibility that polyamines are involved in morphological modification of gills in *F. grandis* after hypoosmotic exposure.
Another key enzyme involved in polyamine biosynthesis is arginase, which exists as at least two different isozymes: arginase I (Arg I) and arginase II (Arg II). Arg I has been observed in diverse groups of organisms to catalyze the catabolism of arginine to form ornithine and urea (Wright et al., 2004). Arg I and Arg II have unique functions based on differential tissue and subcellular distributions. Arg I exists abundantly in the liver of ureagenic species, where it helps catalyze the reaction of the ornithine urea cycle (OUC). Arg II is involved in numerous physiological functions including glutamate, proline, and polyamine biosynthesis, or in the modulation of nitric oxide synthesis in various organs (Morris, 2004; Li et al., 2001). Although in most mammals, arginase I is localized to the cytoplasmic compartment, it is expressed in mitochondria of teleost fish (Wu et al., 1998). However, arginase II is more highly expressed in the mitochondria of extrahepatic tissues. Most teleost fish are obligate ammonioteles lacking a functional OUC beyond embryogenesis; thus, Arg II is the major functional arginase in them to produce ornithine. The current study indicates that the mRNA level and enzymatic activity of arginase are upregulated in *F. grandis* during (Figures 2.1 and 2.2). Arginase activity will constitute the actions of both arginase I and arginase II, despite the fact that only the latter isoform is likely involved in the formation of ornithine for polyamine biosynthesis (Wright et al., 2004).

Exposure of *F. heteroclitus* (Whitehead et al., 2011; Whitehead et al., 2013) and *F. grandis* to fresh water has been shown to strongly upregulate gill ornithine decarboxylase mRNA levels. This study demonstrates that *odc* mRNA and Odc activity were highly inversely correlated with water salinity, and that these effects occurred concomitant with an increase in gill polyamines. In contrast, the marine species
Fundulus majalis, which is intolerant to fresh water, exhibits limited capacity to upregulate odc mRNA levels during hypoosmotic exposures (Whitehead et al. 2013). This pattern suggested that the transcriptional changes in key enzymes of polyamine biosynthesis corresponded to the osmoregulatory abilities within Fundulus species. Moreover, the difference in polyamine level is likely to contribute to species-specific osmoregulatory variation in Fundulus (Whitehead et al., 2011; Whitehead et al., 2013). The roles of polyamines may be related to regulation of cell volume and tissue plasticity in euryhaline fish.

2.4.2 Effects of hypoosmotic exposure on gill polyamine levels

This study suggests that hypoosmotic exposure stimulates polyamine biosynthetic pathways, resulting in an increase in putrescine, spermidine and spermine concentrations in the gill (Figure 2.3). Similar results have been found in brine shrimp (Watts et al., 1996) and mammalian cells (Munro et al., 1975). The relative abundance of the different classes of polyamines may result in the differences in physiological functions during osmosis challenge. Putrescine, spermidine, and spermine concentrations increased in plants (Campestre et al., 2011) and in the gills of the crab Callinectes danae (Silva et al., 2008) with hyperosmotic exposure. Moreover, putrescine and spermidine concentrations in the gills of Callinectes sapidus were also elevated after exposure to full-strength seawater (Lovett and Watts, 1995). In contrast, putrescine concentrations in Artemia nauplii (Watts and Good, 1994) and in Eschericia coli (Munro et al., 1972) decreased with an increase in environmental salinity. Only putrescine level was increased in brine shrimp during hypoosmotic exposure (Watts et al., 1996). Although their functions are not clear, polyamines on one hand are protective of cellular function but on the other hand are
cytotoxic. Thus, regulation of polyamine homeostasis in the face of environmental stressors such as osmotic challenges is paramount. Moreover, the rapidity of changes in polyamine concentrations may play an important role in cell volume regulation because they are responsible for initial efflux of intracellular inorganic ions and intracellular organic compounds (Watts et al., 1996). Further research is necessary to elucidate the specific relationship between polyamines and cell volume regulation. The high ratio of the level of putrescine to the sum levels of spermidine and spermine (Table 2.2) suggested polyamine catabolism regulated the ratio of different polyamine components, and resulted in putrescine accumulation (Adibhatla et al., 2002). Although the role of putrescine during hypoosmotic challenges has not yet been determined, previous studies have shown that putrescine regulates protein transcription (Rhee et al., 2007) and remolds microtubules in mammals (Mechulam et al., 2009). Thus, it is plausible to suggest that putrescine is involved in the gill remodeling, but further studies are needed.

2.4.3 Effects of DFMO on polyamine contents during the hypoosmotic exposure

DFMO is a cancer chemopreventive agent that is used for the prevention of various types of cancer due to its inhibition of polyamine synthesis (Nie et al., 2005), but the physiological effects of DFMO other than on Odc activity are uncertain. DFMO can covalently bind and inactivate Odc (McCann and Pegg, 1992), and thus reduce polyamine levels (Hunter et al., 1990; Bhattacharya et al., 2009). This study showed that the efficacy of DFMO on Odc activity was dependent on exposure salinity. For instance, DFMO significantly reduced the ability of freshwater exposure to stimulate Odc activity in killifish gills, leading to a concomitant reduction in polyamine levels in the tissue (Table 2.3). In comparison, DFMO had no effect on endogenous Odc activity in fish.
gills in the absence hypoosmotic exposure (Table 2.3). Surprisingly, gills still exhibited a marked reduction in gill polyamine concentrations with DFMO despite the inability to inhibit the enzyme. DFMO-sensitive decarbolxylase was detected in IEC-6 cells (Bhattacharya et al., 2009) in human melanoma cells (Kramer et al., 2001) and human malignant glioma cells (Hunter et al., 1990). Moreover, the presence of a DFMO-insensitive decarboxylase was detected in brine shrimp in response to altered salt concentrations (Watts et al., 1996). DFMO-insensitive decarboxylase may lack critical residues for binding DFMO that limit the ability for inhibition by DFMO (Jhingran et al., 2003). Although the reason for this discrepancy is unclear, there are two possible explanations for this reduction of polyamine despite no inhibition of Odc activity. These changes in Odc activity are more likely due to the changes occurring in cellular polyamine contents when DFMO is administered. Polyamines exert an inhibitory effect on Odc synthesis, and previous studies have shown that putrescine, spermidine, and spermine inhibited Odc activity to 85%, 46%, and 0% of the levels in the control group, respectively, in intestinal epithelial crypt (IEC-6) cells (Iwami et al., 1990). Moreover, polyamines stimulated Odc degradation by inducing the synthesis of antizyme (Heby and Persson, 1990; Svensson and Persson, 1996). In this study, intracellular polyamine levels are much lower in 5 ppt-acclimated fish than 0.1-acclimated fish after DFMO treatment (data not shown). The polyamine-mediated feedback regulation of Odc is attenuated in 5 ppt -acclimated fish; thus, Odc activity may keep the original level in 5 ppt -acclimated fish. Another explanation is the existence of DFMO-insensitive decarboxylase. Although there is no direct evidence of a DFMO-insensitive Odc in the gills, the relative percentages of DFMO-sensitive Odc and DFMO-insensitive Odc can be made by looking
at the total Odc activity and the amount inhibited by DFMO. The insensitivity towards DFMO may due to substitution of key substrate binding residues in active site pocket (Preeti et al., 2013). The activity of DFMO-insensitive Odc is one component of total Odc in the gills, but its activity was not affected by DFMO application. However, the data in the current study are insufficient to know the function of DFMO-insensitive decarboxylase during osmotic challenge. The existence of DFMO-insensitive decarboxylase may be involved in the general catabolic reduction of the intracellular amino acid pool that occurs at low salinity (Watts et al., 1996). Moreover, its occurrence may represent an alternative pathway for polyamine biosynthesis (Watts et al., 1996). Further studies are necessary to determine the nature and role of this enzyme.

Additional studies are needed to explore the role of polyamines during the initial shock to and recovery from hypoosmotic exposure in F. grandis. Previous results suggest that there may be a close relationship between Odc expression, polyamines levels and osmotic challenges, although the existence of DFMO-insensitive Odc in killifish complicates this relationship. Although the mechanism is unclear, polyamines may be involved in gill remodeling and acclimation to environmental salinity fluctuation. However, the fact that putrescine, not spermidine or spermine, increased after prolonged freshwater exposure suggests that polyamine catabolic pathways may be upregulated over time due to hypoosmotic shock.
CHAPTER 3: THE POTENTIAL ROLE OF POLYAMINES IN GILL EPITHELIAL REMODELING DURING EXTREME HYPOOSMOTIC CHALLENGES IN THE GULF KILLIFISH

3.1 Introduction

The Gulf killifish, *F. grandis*, and its sister taxa, *F. heteroclitus*, reside in coastal marshes that are subject to frequent and episodic oscillations in salinity, dissolved oxygen, and temperature (Marshall, 2003; Wood and LeMoigne, 1991). Their ability to tolerate these dynamic habitats has made them a widely accepted model for studying the physiological basis of tolerance to environmental stressors (Burnett *et al.*, 2007). Although they prefer brackish to marine salinities (Kaneko and Katoh, 2004; Wood and Grosell, 2008; Wood and Grosell, 2009), euryhaline *Fundulus* species can tolerate reduced environmental salinity and can take short-term forays into fresh water for feeding (Marshall, 2003). Killifish appear to cope with short bouts of fresh water by making rapid, yet easily reversible physiological adjustments (Marshall, 2003; Wood and Grosell, 2008; Wood and Grosell, 2009); only with prolonged exposure to fresh water are they required to make more substantial compensatory adjustments to the physiology of their ion-transporting epithelia (Wood and Grosell, 2008).

One of the most important organs in fish osmoregulation is the gill, which consists of several cell types that mediate the active excretion of salts in marine fish and the active uptake of osmolytes in freshwater fish (Chang *et al.*, 2001; Evans, 2011; Evans *et al.*, 2005). It is generally accepted that the gill epithelium has the ability to remodel itself in response to environmental and physiological perturbations; however, few studies have delineated the salinity at which the gill epithelium transitions from a seawater to a freshwater morphology (Laurent *et al.*, 2006; Katoh *et al.*, 2001; Katoh and Kaneko, 2003). Most *Fundulus* species are marine teleosts that have the ability to retain their
seawater physiology at salinities approaching fresh water (Feldmeth and Waggoner, 1972; Griffith, 1974; Whitehead, 2010). Copeland and Philpott suggested that *F. heteroclitus* made this physiological transition at a salinity between 2 ppt and 0.2 ppt based on a morphological assessment of the fish gill surface (Copeland, 1950; Philpott and Copeland, 1963). Wood and Grosell (2009) reported that the transepithelial potential, an important determinant of the electrochemical driving force of gill ion transport, reversed in polarity instantaneously following acute transfer of seawater-acclimated *F. heteroclitus* to fresh water, but that only with prolonged exposure to fresh water did the gills of *F. heteroclitus* functionally “switch” into one characteristic of a freshwater fish (Wood and Grosell, 2008; Wood and Grosell, 2009). Recently, it has been shown that *F. heteroclitus* acclimated to sea water could be transferred abruptly to 0.4 ppt with no significant effects to their plasma osmolyte concentrations over 14 d post-transfer (Whitehead *et al*., 2012). Although the ability of the gill to remodel with varying environmental salinities is well reported, few investigations have described the cellular mechanisms regulating the transient and long term responses of the fish gill to osmotic challenges.

It has recently been shown that the upregulation of transcripts encoding enzymes involved in polyamine biosynthesis is an early compensatory response to hypoosmotic exposure in the *F. heteroclitus* gill (Whitehead *et al*., 2011). Polyamines are a family of organic cations in cells, and their accumulation are affected by environmental salinity in bacteria (Peter *et al*., 1978), plants (Zapata *et al*., 2004) and mammalian cells (Poulin *et al*., 1991). Polyamines, which include the multi-aminated molecules, putrescine, spermidine, and spermine, increase in concentration in the killifish gill during hypoosmotic challenge (0.1 ppt), a response associated with increased transcription and
enzymatic activity of ornithine decarboxylase, the rate-limiting step that converts ornithine to putrescine (Chapter 2). Following 3 d of acute transfer from sea water to fresh water, only gill putrescine levels remained upregulated in killifish, suggesting a distinct role of this polyamine in freshwater acclimation or in the maintenance of the freshwater gill phenotype; still, little is known of the physiological function of these molecules in fish.

The main goal of this study was to characterize the role of polyamines in osmoregulation, gill remodeling, and apoptosis in the Gulf killifish, *Fundulus grandis*, during exposure to a highly-resolved range of low environmental salinities. This salinity range was chosen because it was known to demarcate the transition between the marine and freshwater gill osmoregulatory phenotypes (Feldmeth and Waggoner, 1972; Griffith, 1974; Whitehead, 2010; Marshall, 2003). The effects of alpha-DL-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase, on plasma chemistry and the expression of various apoptotic markers in the fish gill during freshwater transfer were studied. Finally, the effects of polyamine supplementation on active ion transport and membrane conductance in the opercular epithelium under hypoosmotic conditions were studied. The opercular epithelium has been used as a surrogate model of the marine fish gill (Zadunaisky et al., 1995; Zadunaisky, 1996) to study the mechanisms of cell volume regulation during osmotic challenges (Marshall et al., 2005).
3.2 Materials and methods

3.2.1 Experimental animals

Adult *F. grandis* were obtained from a local hatchery (Gulf Coast Minnows, Thibodeaux, LA) and kept in the Life Sciences Aquatic Facility at Louisiana State University (Baton Rouge, LA, USA). Fish were maintained in 5 ppt water for at least one month in a 570-liter glass aquarium with mechanical and biological filtration, and UV-sterilization. Water salinity was monitored using a water quality meter (YSI, Yellow Spring, OH, USA) and temperatures were kept at 22-24 °C. Fish were kept on a light cycle of 12 h light and 12 h dark provided by fluorescent lighting set on an automatic timer. Fish were fed a commercial fish pellet (Cargill, Aquaxell™ WW Fish Starter 4512, Franklin, LA, USA) twice daily at 2% body weight throughout experimentation, and partial water changes were performed at least twice per week to keep water quality constant.

3.2.2 Experimental protocols

Series 1: Blood plasma chemistry and gill surface morphology were assessed during acute hypoosmotic challenges. One hundred and fifty adult fish (weight range 4.3-16.8 g) were acclimated to 5 ppt water for at least one month. Immediately prior to sampling, a subset of fish (N=6) were sampled as described below. The remaining fish were divided randomly into 5 groups, then transferred acutely to 5, 2, 1, 0.5, or 0.1 ppt water, which was made by diluting Instant Ocean® salt (Blacksburg, VA, USA) with reverse osmosis water. At 6 h, 1 d, 3 d, or 7 d post-transfer (N = 6 per salinity per time point), fish were net-captured and anesthetized with 0.5 g/L tricaine methanesulfonate (MS-222). Whole gill baskets were dissected from fish and washed with MilliQ-purified
water for 10 s. The 3\textsuperscript{rd} and 4\textsuperscript{th} left gill arches were fixed in a solution of 2\%
glutaraldehyde and 1\% formaldehyde in 0.1 M cacodylate buffer for scanning electron
microscopic (SEM) analysis of the gill surface, as described in Section 3.2.3. Blood was
collected into micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA, USA)
following caudal severance and centrifuged at 3,000 g for 3 min. Plasma was collected
and stored at -20 °C while awaiting analysis of plasma osmolality, as described in Section
3.2.4.

Series 2: Previous studies have shown that the mRNA abundance and enzymatic
activity of ornithine decarboxylase is significantly upregulated in gills of \textit{F. grandis} post-
transfer to 0.1 ppt water (Chapter 2). It has also been established that inhibition of
ornithine decarboxylase during hypoosmotic challenge significantly reduces the
concentrations of all three major polyamines in the gill (Chapter 2). In these studies, the
influence of inhibition of ornithine decarboxylase on plasma osmolality, plasma Na\textsuperscript{+} and
Cl\textsuperscript{-} concentrations, and caspase-3 activity were assessed during hypoosmotic exposure.
One hundred and twenty adult \textit{F. grandis} (weight range 4.9-12.3 g) were acclimated to 5
ppt water, and fish were weighed and injected intraperitoneally with 10 µL phosphate-
buffered saline (PBS; sham control; 146 mM NaCl, 3 mM KCl, 15 mM NaH\textsubscript{2}PO\textsubscript{4}, 15
mM Na\textsubscript{2}HPO\textsubscript{4}, and 10 mM NaHCO\textsubscript{3} at pH 7.4) or DFMO (0.2 mg DFMO/µL PBS) per g
fish for two days prior to hypoosmotic transfers. At 3 d, half of the fish in the DFMO
and PBS treatments were transferred to 0.1 ppt water and the other half of the remaining
fish were transferred to 5 ppt water. Fish were injected daily with either DFMO or PBS,
as described above. Plasma was collected using the same procedure as described for
Section 3.2.2 (series 1) and stored at -20 °C while awaiting analysis of plasma chemistry,
as described in Section 3.2.4. All four gill arches from the right side were flash frozen in liquid nitrogen and stored at -80 °C for analysis of caspase-3 activity, as described in Section 3.2.6.

Series 3: Considering the extensive remodeling of the gill epithelium (Figure 3.2) and the significant increase in transcripts encoding enzymes involved in polyamine biosynthesis (arginase II and ornithine decarboxylase) (Chapter 2), the effects of salinity challenges on the mRNA levels of arginase II (*arg II*) and ornithine decarboxylase (*odc*) in isolated mitochondrion-rich cells (MRCs) and pavement cells (PVCs) of the gill epithelium were investigated. Forty-eight fish (weight range 8.19-17.83 g) were acclimated in 5 ppt water for at least one month. Half the fish were transferred to 5 ppt water and the other twenty-four fish were transferred to 0.1 ppt water. After one day post-transfer, fish were sacrificed and their gills removed. MRCs and PVCs were isolated from pooled fish gills (i.e., 2 gills per isolation) using the procedure described in Galvez *et al.*, 2002, with modifications described below. Briefly, *F. grandis* fish gills were washed three-times in ice-cold phosphate-buffered saline (137 mM NaCl, 4.3 mM Na$_2$HPO$_4$, 2.7 mM KCl, and 1.4 mM NaH$_2$PO$_4$ at pH 7.8) for 10 min. Gill filaments were removed from their arches, cut into small sections, and digested in 0.25% Trypsin-EDTA (Sigma, St. Louis, MO, USA) for 20 min at 300 RPM using a SK-300 shaker (Lab companion, Geumcheon-Gu, Seoul, Korea). Gill digests were passed through a 96 µm mesh filter in 15 mL stop buffer (Kelly *et al.*, 2000). Gills were trypsin-digested 2 or 3 times to yield a mixed population of isolated gill cells, then centrifuged at 300 g for 8 min at 4 °C. Gill cells were diluted in a 10-fold volume of PBS centrifuged at 500 g for 10 min at 4 °C. The pellet, which was resuspended in 2 mL PBS, was applied to the top of a
discontinuous Percoll gradient (Sigma, St. Louis, MO, USA) consisting of a 1.03 g/ml top layer, a 1.06 g/ml middle layer, and a 1.09 g/ml bottom layer. The stacked cell suspension was centrifuged (1,000 g for 35 min at 4 °C) to isolate PVCs and MRCs at the 1.03-1.06 g/mL and 1.06-1.09 g/mL Percoll interfaces, respectively. Each isolated cell fraction was stored at -80 °C until RNA extraction, then analyzed by quantitative PCR for *arg II* and *odc* mRNA levels, as described in Section 3.2.5.

Series 4: Tests were conducted to assess the putative role of polyamines in the compensatory response of fish to osmotic shock. The opercular epithelium was used as a surrogate of the fish gill to study the influence of polyamines on membrane conductance (*G*<sub>t</sub>, mS cm<sup>-2</sup>) and short-circuit current (*I*<sub>sc</sub>, µA cm<sup>-2</sup>) (Marshall et al., 2000). Adult *F. grandis* were acclimated to 5 ppt water and anaesthetized in 0.2 g/L MS 222. Both opercular membranes of the fish were isolated as described by Marshall et al. (2000). Briefly, each operculum was mounted between two plexiglass sliders (Physiological instruments, San Diego, CA, USA), exposing a 0.1 cm<sup>2</sup> diameter round aperture, and placed in individual Ussing chambers (Physiological instruments, San Diego, CA, USA) (Marshall et al., 2000). The Ussing chambers contained isotonic saline (151 mM NaCl, 3 mM KCl, 0.88 mM MgSO<sub>4</sub>, 4.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.48 mM K<sub>2</sub>HPO<sub>4</sub>, 1.0 mM CaCl<sub>2</sub>, 11.0 mM HEPES-free salt, and 4.5 mM urea at an osmolality of 334 mOsm/kg and pH 7.8) on each side of the membrane, as described previously (Daborn et al., 2001). The isotonic bathing solution was bubbled with 100% oxygen. The basolateral side of the membrane was bathed in 2 mL isotonic saline with 3 mM glucose added, whereas the apical side contained 2 mL isotonic saline. *I*<sub>sc</sub> and *G*<sub>t</sub> across the opercular epithelium were measured in real time with a data acquisition system (PowerLab and LabChart 7 software,
ADInstrument Inc., CO, USA). A pulse of 0.002 V was given every min to calculate \( G_t \) according to Ohm’s law: \( G_t = I_{sc} / V \). Once a baseline was achieved under symmetrical conditions (isotonic saline on both sides of the membrane), the isotonic solution was replaced with a hypotonic bathing solution (75% isotonic saline, 25% distilled water) under symmetrical conditions, resulting in an osmolality of 270 Osm/kg. This hypotonic solution mimicked the plasma osmolality of killifish during the early stages of hypoosmotic shock.

\( I_{sc} \) and \( G_t \) were assessed in opercula immersed in isotonic (Iso) and hypotonic (Hypo) bathing solutions for 30-45 min each to allow steady-state values to be reached. Preliminary experiments were conducted to assess the influence of polyamine concentration on \( I_{sc} \) during hypotonic shock. Briefly, baseline \( I_{sc} \) for opercular epithelium exposed to Iso and Hypo solutions were determined. Individual polyamines at final concentrations of 0.4-400 mM putrescine, 0.3-300 mM spermidine, and 0.1-100 mM spermine (N=6 per concentration per polyamine) were added to the basolateral side of opercular epithelium (V:V of 1:1000) and \( I_{sc} \) was recorded until it reached a new steady state (approximately 25-30 min). These data were used to derive the relationship between changes in \( I_{sc} \) versus polyamine concentrations for each of the three compounds (data not shown). Since there was no significant difference in change in \( I_{sc} \) change between low dose and high dose of individual polyamines, a polyamine mixture consisting of 0.4 mM putrescine, 0.3 mM spermidine, and 0.1 mM spermine was used in subsequent experiments as described below. First, the influence of polyamine mixture on the change of \( I_{sc} \) and \( G_t \) was assessed. Briefly, a steady state of \( I_{sc} \) and \( G_t \) was attained under Iso condition, then a polyamine mixture (0.4 mM putrescine, 0.3 mM spermidine,
0.1 mM spermine) was added to the basolateral side of opercular epithelia, either a) coincident with transfer from isotonic to hypotonic conditions (pre-PA, N=7), or b) following initial hypotonic transfer and establishment of a steady-state $I_{sc}$ under hypotonic conditions (post-PA, N=10). Second, the influence of individual polyamines on the change of $I_{sc}$ and $G_i$ was assessed. 0.4 mM putrescine (PUT), 0.3 mM spermidine (SPD), and 0.1 mM spermine (SP) were added to the basolateral side of opercular epithelia coincident with transfer from isotonic to hypotonic conditions, respectively (N=7 for Hypo+PUT, Hypo +SPD and Hypo +SP). $I_{sc}$ and $G_i$ were recorded for 40 min until reaching a steady-state, and percent change of $I_{sc}$ and $G_i$ was measured according to Equation 3.1,

$$\text{Percent change} = \frac{\text{Value}_{\text{treat}} - \text{Value}_{\text{isotonic}}}{\text{Value}_{\text{isotonic}}}$$

where $\text{Value}_{\text{treat}}$ represents the values of $I_{sc}$ or $G_i$ at pre-PA, post-PA, Hypo, Hypo+PUT, Hypo +SPD and Hypo +SP at different time points. $\text{Value}_{\text{isotonic}}$ indicates a steady-state of $I_{sc}$ or $G_i$ when opercular epithelia were incubated in isotonic saline.

Series 5: Killifish gill $c$-fos and $c$-myc mRNA transcript levels and caspase-3 activity were assessed as putative measures of cell proliferation and cell apoptosis following acute freshwater transfer. Sixty adult fish (weight range 3.83-12.61 g) were acclimated to 5 ppt water for at least one month. Gills were sampled prior to transfer and at 6 h, 1 d, 3 d, and 7 d post-transfer to 5 ppt or 0.1 ppt water (N = 6 per salinity per time point). The first two left gill arches were immersed in RNAlater and stored at 4 °C overnight, then transferred to -20 °C while awaiting total RNA extraction and qPCR analyses of $c$-fos and $c$-myc transcript abundance, as described in Section 3.2.5. All four
gill arches from the right side were flash frozen in liquid nitrogen, stored at -80 °C, and used for analysis of caspase-3 activity, as described in Section 3.2.6.

3.2.3 Scanning electron microscopy (SEM) analyses

Gills were fixed in 2% glutaraldehyde and 1% formaldehyde in 0.1 M cacodylate for 4 h, rinsed in 0.1 M cacodylate buffer containing 0.02 M glycine, post-fixed in 2% osmium tetroxide for 1 h, and rinsed in water. Samples were dehydrated in an ethanol series (10 min at 50%, 10 min at 70%, 10 min at 80%, 2 rinses of 10 min at 95%, and 3 rinses of 15 min at 100% ethanol), dried with liquid CO₂ in a Denton critical point dryer (Cherry Hill, NJ, USA,) mounted on aluminum SEM stubs and coated with a gold:palladium solution (V:V of 60:40) in an Edwards S150 sputter coater (Ashbord, Kent, UK). The afferent edge of each gill filament was randomly imaged using a JSM-6610 high vacuum mode SEM (Tokyo, Japan).

3.2.4 Plasma chemistry analyses

Plasma osmolality was analyzed by freeze-point depression (Precision systems INC, Natick, MA, USA). Plasma Na⁺ concentrations were measured using flame atomic absorption spectroscopy (Varian Australia Pty Ltd, Australia), and plasma Cl⁻ concentrations were measured using a modified mercuric thiocyanate method (Zall et al., 1956).

3.2.5 Relative arg II, odc, c-myc, and c-fos mRNA levels

Commercial SYBR Green master mix (QIAGEN Inc. Valencia, CA, USA) was used to analyze the relative mRNA levels of arg II, odc, c-myc, c-fos, and the reference gene, 18 S ribosomal RNA (18s rRNA). Expressed sequence tag (EST) sequences for arg II (AB290198.1), odc (CN977303.1), c-fos (DN956552.1) and c-myc (CN985702.1) from
F. heteroclitus were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). Primers for quantitative PCR were developed for all genes using Integrated DNA Technologies Inc. software (Coralville, IA, USA) (Table 3.1). A 20 μL reaction mixture (0.1 μg cDNA, 10 nM forward and reverse primers, 5 μL SYBR Green reagents) was used for the ABI Prism 7000 SDS system (Carlsbad, CA, USA). The first strand of cDNA was reverse-transcribed from total mRNA of MRCs or PVCs isolated from the fish gill. Samples were cycled 40 times at 95 °C for 15 s and 60 °C for 1 min. Efficiency and relative changes in mRNA levels were measuring using the $2^{-\Delta\Delta Ct}$ method (Fleige et al., 2006).

Table 3.1. Sequences of forward and reverse primers used for quantitative RT-PCR analyses of c-myc, c-fos, arg II, odc, and 18s rRNA amplicons in F. grandis gill.

<table>
<thead>
<tr>
<th>Name</th>
<th>Direction</th>
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</tr>
<tr>
<td>c-myc</td>
<td>Reverse</td>
<td>AGCCTTCGAAATCCTCCACGTTCTT</td>
</tr>
<tr>
<td>c-fos</td>
<td>Forward</td>
<td>ATCTGACAGCATCAAGTGCTCCT</td>
</tr>
<tr>
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<td>Reverse</td>
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</tr>
<tr>
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<tr>
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<tr>
<td>18s rRNA</td>
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3.2.6 Caspase-3 activity assay

A caspase-3 assay kit (Sigma, St. Louis, MO, USA) was employed to measure caspase-3 activity, which is based on the hydrolysis of acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin by caspase 3 that results in the release of the fluorescent 7-amino-4-methylcoumarin (AMC). Gill arches were homogenized with a 10-fold volume of 0.85% NaCl, and the original homogenate was used directly to measure caspase-3 activity. Original gill homogenates were diluted 20-fold and measured for total protein (Sigma, St. Louis, MO, USA). Fluorescence intensity was measured with excitation at 355 nm and emission at 460 nm using a VICTOR™ X3 multilabel plate reader (Perkin Elmer, Santa Clara, CA, USA). A 1x assay buffer of 200 µl served as a blank to adjust the fluorimeter to zero. Fold–change in gill caspase-3 activity was calculated by taking the ratio of the enzymatic activity of post-transfer to 5 ppt water or 0.1 ppt water over the enzymatic activity of pre-transfer at each time point.

3.2.7 Statistical analysis

Statistical differences in plasma osmolality, gene transcripts, and $I_{sc}$ and $G_t$ between treatments were analyzed using a one-way ANOVA (v.9.3, SAS Institute Inc., Cary, NC, USA). Statistical differences were considered significant at a $P$-value of 0.05 or less.

3.3 Results

3.3.1 Plasma chemistry

Initial experiments were performed to assess the influence of comparably small changes in environmental salinity on plasma osmolality (Figure 3.1) and gill surface morphology (Figure 3.2). The magnitude of the reduction of plasma osmolality
disturbances depended on the severity of the hypoosmotic transfer and on the time following post-transfer. Plasma osmolality decreased by approximately 19.5% following transfer from 5 ppt water to either 0.1 ppt or 0.5 ppt water, reaching their lowest values at 1 d and 6 h, respectively (Figure 3.1). Plasma osmolality in the 0.5 ppt treatment recovered to its original level by 1 d, but it did not completely recover to the pre-transfer value even after 7 d in the 0.1 ppt treatment ($P=0.0069$). In comparison, plasma osmolality in *F. grandis* was not affected by the abrupt transfer from 5 ppt to 1 ppt, 2 ppt, or 5 ppt water at any time during the 7 d post-transfer period.

![Figure 3.1: Plasma osmolality (N = 6) in *F. grandis* acclimated to 5 ppt water then sampled at pre-transfer or at 6 h, 1 d, 3 d, and 7 d following acute transfer to 0.1, 0.5, 1, 2, and 5 ppt water. The vertical dashed line divides pre-transfer and post-transfer time points. Data represent the mean ± SEM, mOsM/kg. Asterisks indicate a significant difference ($P \leq 0.05$) in comparison to the 5 ppt treatment for that time point; different letters indicate a significant difference ($P \leq 0.05$) in comparison to the pre-transfer for a given salinity.](image-url)
3.3.2 Gill morphology

Scanning electron microscopy was used to assess the morphology of the apical opening of MRCs on the gill surface of fish undergoing osmotic shock, as described in Section 3.2.3. The gill surface of fish acclimated to salinities of 1-5 ppt water consisted of pavement cells (PVCs) possessing concentric rings of microridges around the outer cell margins, and, on occasion, microvilli projecting towards the center of cells (Figure 3.2).

Figure 3.2: Representative scanning electron micrographs of the afferent filamental epithelium of *F. grandis* gills after acclimation to 5 ppt and acute transfer to 0.1, 0.5, 1, 2, or 5 ppt water at 6 h, 1 d, 3 d, and 7 d post-transfer. Asterisks indicate pavement cells, solid white arrows point to seawater-type mitochondrion-rich cells (MRCs), and dashed white arrows point to freshwater-type MRCs. Scale bar = 5 μm.
MRCs in fish gills exposed to 5 and 2 ppt water were predominately seawater-type, as noted by their small apical openings at the gill surface which were localized to the afferent edge of the filamental epithelium. Exposure to 1 ppt water led to minor changes in the surface appearance of the fish gill, with a slight enlargement of MRCs during the initial 6 h to 1d post-transfer. FW-type MRCs, characterized by their convex shape and well-developed microvilli, began to emerge on the gill surface as salinity decreased to 0.5 ppt and below. These cell types were only expressed transiently, being replaced by SW-type MRCs, which reemerged by 3 d at 0.5 ppt or by 7 d at 0.1 ppt. Regardless, FW-type MRCs with smaller apical openings were still present following 7 d exposure to 0.1 and 0.5 ppt water (Figure 3.2).

3.3.3 *argII* and *odc* mRNA levels in pavement cells and mitochondrion-rich cells

Considering the significant increase in *arg II* and *odc* mRNA levels in the gill during freshwater acclimation (Chapter 2), the cellular distribution of these increases in transcript abundance was assessed. Compared to the PVCs at 5 ppt, the levels of *arg II* and *odc* mRNA in MRCs did not change. In contrast, *arg II* and *odc* mRNA levels increased by 11.4-fold (*P*=0.0037) and 8.1-fold (*P*=0.0009) in MRCs upon transfer of fish to 0.1 ppt water (Figure 3.3). During hypoosmotic exposure, *arg II* (Figure 3.3 panel A) and *odc* (Figure 3.3 panel B) mRNA levels were 4.9-fold (*P*=0.0224) and 2.6-fold (*P*=0.0113) higher in MRCs relative to PVCs, respectively.

3.3.4 Plasma chemistry during ornithine decarboxylase inhibition

Compared to the pre-transfer level, plasma osmolality declined approximately 20% after 1 d hypoosmotic transfer in the sham control (*P*=0.0039). In contrast, fish
Figure 3.3: Relative arg II and odc mRNA levels in mitochondrion-rich cells (MRCs) and pavement cells (PVCs) in gills of fish acclimated to 5 ppt, then acutely transferred to 5 ppt or 0.1 ppt water for 1 d. The mRNA levels were normalized to the mean value of expression in PVCs from 5 ppt-acclimated fish. Data represent the mean ± SEM (N=6). Different letters indicate a significant difference (P≤0.05) in comparison to the 5 ppt treatment for the same cell type; asterisks indicate a significant difference (P≤0.05) in comparison to PVCs for that salinity.

administered DFMO, showed no effect of freshwater transfer over time (Figure 3.4 panel A). Plasma osmolality was not significantly different between the DFMO-treated fish and PBS-treated fish maintained in 5 ppt water over time (Figure 3.4 panel A). Plasma Na⁺ concentrations decreased by 14% at 6 h and 18% at 1 d following transfer to 0.1 ppt water hypoosmotic exposure in sham controls, but returned to the pre-transfer level by 3 d (Figure 3.4 panel B). The plasma Na⁺ loss induced within the first day of by freshwater exposure (0.1 ppt) was attenuated by administration of DFMO (Figure 3.4 panel B), although it returned to the pre-transfer level by 3 d. Compared to the pre-transfer level, plasma Cl⁻ concentration decreased at 6 h and 1 d following hypoosmotic exposure in sham controls. DFMO application decreased the reduction of plasma Cl⁻ induced by hypoosmotic exposure at 6 h and 1 d (P=0.0084 and 0.016, respectively) (Figure 3.4
panel C). Both plasma Na\(^+\) and Cl\(^-\) returned to pre-transfer levels after 3 d of hypoosmotic exposure in shams. Moreover, the concentrations of plasma Na\(^+\) and Cl\(^-\) in DFMO and PBS-treated fish at 5 ppt water were not significantly different over time (Figure 3.4 panel B and C).

![Graphs showing plasma osmolality, sodium, and chloride concentrations over time.](image)

Figure 3.4: Plasma osmolality (mOsM/kg) (A), plasma sodium concentration (mM) (B), and plasma chloride concentration (mM) (C) in *F. grandis* acclimated to 5 ppt and sampled at pre-transfer or at 6 h, 1 d, 3 d, and 7 d following acute transfer to 0.1 ppt or 5 ppt water. Fish received intraperitoneal injections of DFMO or PBS (sham control) (see text for details). The vertical dashed line divides pre-transfer and post-transfer. Data represents the mean ± SEM (N=6). Asterisks indicate a significant difference (\(P \leq 0.05\)) in comparison to the 5 ppt PBS treatment for that time point; letters indicate a significant difference (\(P \leq 0.05\)) in comparison to the pre-treatment for a given salinity.
3.3.5 The effect of polyamines on opercular $I_{sc}$ and $G_t$

The influence of polyamines on the hypotonic-induced changes in $I_{sc}$ and $G_t$ in the opercular epithelium was investigated. $I_{sc}$ of the opercular epithelium dropped immediately after transfer to the hypotonic solution, reaching a new steady state within 30-35 min (Figure 3.5 panel A). Hypotonic shock induced a 52% reduction in $I_{sc}$ compared to that measured in the isotonic medium (Figure 3.5 panel A). Compared to the hypotonic control, pre-PA significantly inhibited $I_{sc}$ reduction in the opercular epithelium after 20 min hypotonic exposure, reaching its maximum inhibition at 40 min ($P=0.0006$) (Figure 3.5 panel A). However, post-PA did not affect $I_{sc}$ compared to the hypotonic control over time (Figure 3.5 panel A). Hypotonic shock immediately decreased $G_t$, which could not be inhibited by either pre-PA or post-PA application over time (Figure 3.5 panel B).

Additional experiments were conducted to assess the contribution of individual polyamines to changes in $I_{sc}$ and $G_t$ during hypotonic shock. $I_{sc}$ and $G_t$ of the opercular epithelium dropped immediately after transfer to the hypotonic solution, reaching new steady states within 30-35 min (Figure 3.6 panel A). Compared to the hypotonic control (52% decrease), only spermidine inhibited the reduction of $I_{sc}$ and $G_t$ over time, having its maximal effect at 40 min ($P<0.01$) (Figure 3.6). Hypotonic shock significantly decreased $G_t$, which was not inhibited by the application of putrescine or spermine (Figure 3.6 panel B).

3.3.6 Relative $c$-myc, $c$-fos mRNA expression, and caspase-3 activity

The mRNA levels for $c$-fos and $c$-myc were measured in the gills following hypoosmotic exposure as a measure of cell proliferation in the tissue. Gill $c$-fos mRNA
Figure 3.5: Percent change (relative to baseline isotonic conditions) in the short circuit current, $I_{sc}$ (A) and membrane conductance, $G_t$ (B) of opercular epithelia kept in symmetrical conditions to isotonic (Iso) (N=10) or hypotonic (Hypo) solutions (N=10). The vertical dashed line represents the transfer between these two media. Controls represent those epithelia receiving no administration of the polyamine mixture. Asterisks represent significant differences from hypotonic control exposures at the same time points ($P<0.05$). A polyamine mixture (0.4 mM putrescine, 0.3 mM spermidine, and 0.1 mM spermine) was added to the basolateral side of opercular epithelium, either a) coincident with transfer from isotonic to hypotonic conditions (pre-PA), or b) following initial hypotonic transfer and establishment of a steady-state $I_{sc}$ under hypotonic conditions (post-PA).
Figure 3.6: Percent change (relative to baseline isotonic conditions) in short circuit current, $I_{sc}$ (A) and membrane conductance, $G_t$ (B) of the opercular epithelium in isotonic (Iso) or hypotonic (Hypo) solutions. The vertical dashed line represents the transfer between these two media. Data represents the mean ± SEM (N=6). Controls represent those epithelia receiving no addition of polyamines. Single asterisks represent significant differences from hypotonic challenges at the same time points ($P \leq 0.05$); double asterisks represent significant differences from hypotonic challenges at the same time points ($P \leq 0.01$).
expression increased 14-fold at 6 h ($P<0.0001$) and 4-fold at 1 d ($P=0.001$) post-transfer to 0.1 ppt water, but returned to the pre-transfer level by 3 d (Figure 3.7 panel A). Compared to the 5 ppt control, the *c-fos* mRNA level increased 10.5-fold and 4.4-fold at 6 h and 1 d, respectively, following hypoosmotic exposure ($P<0.02$ for both time points) (Figure 3.7 panel A). Gill *c-myc* mRNA abundance was increased 6.1-fold after 6 h of hypoosmotic exposure, but returned to the pre-transfer level at 7 d (Figure 3.7 panel B). The level of *c-myc* mRNA increased up to 7.1-fold by 1 d post-transfer (compared to the pre-transfer level) ($P<0.0001$) (Figure 3.7 panel B). *c-myc* mRNA expression increased at 6 h, 1 d, and 3 d in the gills of 0.1 ppt-acclimated fish ($P=0.001$, 0.011, and 0.029, respectively) (Figure 3.7 panel B).

Based on the gill remodeling observed in Figure 3.2, the onset of apoptosis was assessed by analyzing caspase-3 activity in gill. Compared to the pre-transfer caspase-3 activity, caspase-3 activity in the gill did not change significantly during exposure to 5 ppt water (Figure 3.7 panel C). However, gill caspase-3 activity increased approximately 3-fold following 3 d of hypoosmotic exposure ($P=0.0062$) and returned to the control level at 7 d (Figure 3.7 panel C). Compared to 5 ppt, hypoosmotic exposure (0.1 ppt) increased gill caspase-3 activity significantly at 1 d ($P=0.0429$) and 3 d ($P=0.0399$) (Figure 3.7 panel C). After hypoosmotic exposure, gill caspase-3 activity was increased at 6 h and 1 d significantly ($P<0.0001$ for both time points) compared to the sham control (Figure 3.7 panel D), and this upregulation was inhibited by 38% at 6 h ($P=0.0002$) and 43% at 1 d ($P<0.0001$) after administering DFMO (Figure 3.7 panel D).
Figure 3.7: Relative c-fos (A) and c-myc (B) mRNA levels (relative to the pre-transfer levels), and caspase-3 activity without DFMO application (C) and with DFMO application (D). The vertical dashed line divides pre-transfer and post-transfer. Data represent the mean ± SEM (N=6). Asterisks indicate significant differences ($P \leq 0.05$) in comparison to the 5 ppt PBS treatment for that time point; letters indicate a significant difference ($P \leq 0.05$) in comparison to the pre-treatment for different time points.

3.4 Discussion

3.4.1 Gill epithelium morphology

In the present study Gulf killfish were acclimated to 5 ppt water (~15 % sea water), then subjected fish to small reductions to environmental salinity. This study highlighted the extent to which only a small reduction in environmental salinity (i.e.,
from 1 ppt to 0.5 ppt) produced a dramatic change in plasma osmotic balance and gill surface morphology. Plasma osmolality was significantly reduced at 6 h post-transfer to 0.5 ppt water, although it recovered back to the 5 ppt control level within 1 d (Figure 3.1). Fish transferred to 0.1 ppt water experienced a similar reduction in plasma osmolality (Figure 3.1), although osmotic balance was not restored to pre-transfer levels even after 14 d exposure (data not shown). The results showed that the apical surface of the gill MRCs of *F. grandis* retained an apical crypt characteristic of a seawater fish gill at salinities of 1 ppt and above (Figure 3.2). Only when acutely exposed to salinities at or below 0.5 ppt did the apical MRCs on the fish gill surface attain a concave appearance more characteristic of a freshwater gill phenotype (Figure 3.2). In fact, gill surface morphology, as determined using SEM (Figure 3.2), was shown to change from its basal SW-type phenotype only in treatments and at time points in which fish had also experienced reductions in plasma osmolality (Figure 3.1). The drastic decrease of plasma osmolality during hypoosmotic exposure was considered to be the result of diffusional ion loss through the body surfaces. When plasma osmolality decreased, the size of MRCs increased as shown in Figure 3.2. It has been demonstrated that MRCs alternate their morphology to meet abrupt environmental osmotic change (Kaneko and Katoh, 2004). Fish exposed to salinities of 0.1 ppt or 0.5 ppt exhibited few seawater-type MRCs after 6 h; these cells were replaced by large MRCs that were predominantly triangular in shape, with apical surfaces that contained short microvilli flush with the surrounding PVCs (Figure 3.2). Laurent (2006) termed these freshwater-type MRCs as cuboidal cells, characterized by their wedge-like appearance between neighboring PVCs, analogous to the mitochondrion-rich PVCs described in rainbow trout (i.e., PNA- MRCs; see Chapter
1). With continued extreme hypoosmotic exposure, the surface exposure of these cuboidal cells decreased over time and seawater-type apical crypts emerged, even in fish acclimated to 0.1 ppt water (Figure 3.2). This produced a mixed population of SW and FW MRCs, as previously described (Laurent et al., 2006; Scott et al., 2006; Scott et al., 2008; Whitehead et al., 2012). It is unclear why SW MRCs began to resurface in the 0.1 ppt- and 0.5 ppt-acclimated fish. Laurent et al. (2006) suggest that seawater MRCs either undergo apoptosis, necrosis, exfoliation, or are simply covered by pavement cells.

SEM images showed a transition between the two morphologies, in which freshwater-acclimated Fundulus lacked the apical cavity characteristics of seawater-acclimated fish (Copeland, 1950; Kaneko and Katoh, 2004; Katoh et al., 2001; Katoh et al., 2000; Laurent et al., 2006). This ability to rapidly transition based on environmental salinity is beneficial for euryhaline fish species, such as F. grandis. One possible mechanism may be related to the regulation the Cl⁻ ion transport across the gill epithelium. In order to acclimate to fresh water, killifish must actively absorb ions such as Na⁺ and Cl⁻ from dilute aquatic environment or inhibit the loss of plasma Na⁺ and Cl⁻ to compensate for diffusive ion loses and osmotic water gain. The freshwater-acclimated fish had cuboidal cells (as observed by scanning electron microscopy) that intercalated between PVCs or other MRCs forming a relatively impermeable barrier to ions (Kaneko et al., 2002; Katoh and Kaneko, 2003), preventing ion loss in the gill epithelium. The morphological transition was also accompanied by the loss of apical chloride channels (cystic fibrosis transmembrane conductance regulator), leading to an inhibition of Cl⁻ secretion (Katoh et al., 2001; Katoh and Kaneko, 2003). In summary, the appearance of
cuboidal cells presumably would help inhibit ion loss at the gill epithelium and maintain plasma ion concentrations in response to rapid fluctuations in environmental salinity.

3.4.2 Possible roles of polyamines in cell apoptosis and cell proliferation

The regulation of cell apoptosis and caspase-3 activity may be related to gill remodeling during compensation to salinity challenges. The present study demonstrated that relative arg II and odc mRNA expression was elevated in the MRCs of 5 ppt-acclimated fish gills and that these transcripts further increased in abundance in this cell type upon 0.1 ppt water exposure (Figure 3.3). Arginase II is abundant in extrahepatic cells rich in mitochondria, where it may be involved in numerous physiological functions including glutamate, proline, and polyamine biosynthesis, or in the modulation of nitric oxide synthesis in various organs (Morris, 2004). The concurrence of increasing arg II and Odc mRNA levels in MRCs (Figure 3.3) and MRC remodeling (Figure 3.2) suggested that polyamines may be associated with the phenotypic plasticity of MRCs in the fish gill. Although the role of polyamines in MRCs remodeling of killifish is unclear, polyamines can cause cell apoptosis (Gerner and Meyskens, 2004), which is thought to mediate gill remodeling in killifish (Laurent et al., 2006). Moreover, hypoosmotic challenges led to a significantly transient increase in gill putrescine, spermidine and spermine concentrations (Chapter 2). Polyamine accumulation occurred concomitantly with an increase in gill caspase-3 activity following 0.1 ppt water exposure (Figure 3.7 panel C). Caspase-3 activity also increased in zebrafish during DNA damage (Lee et al., 2008), in Atlantic salmon (Salmo salar) during hyperthermia (Takle et al., 2006), in sea bass (Dicentrarchus labrax L.) during bacterial infection (Reis et al., 2007), in rainbow trout after hyperosmotic transfer (Rojo and Gonzalez, 1999), and in sturgeon (Acipenser
Schrenckii) during prolonged hypoxia (Lu et al., 2005). The role of caspase-3 in fish may correspond with extensive apoptosis and morphological abnormalities in tissues (Yamashita et al., 2008). The results in the present study indicated that polyamines may activate the intrinsic apoptotic pathway (chapter 1) via caspase-3, as seen in previous studies (Poulin et al., 1995; Stefanelli et al., 1998; Tobias and Kahana, 1993). Additionally, accumulation of caspase-3 during hypoosmotic challenge may be involved in the regulation of tissue plasticity (Snigdha et al., 2012).

The effect of DFMO on caspase-3 activity in the fish gill during freshwater transfer was studied. DFMO application resulted in the reduction of gill polyamine levels during hypoosmotic exposure (Chapter 2). Hypoosmotic challenge increased caspase-3 activity at 6 h and 1 d (Figure 3.7 panel D); however, applying DFMO decreased gill caspase-3 activity, as observed in intestinal epithelia (Deng et al., 2005; Ray et al., 2000), in bone marrow stromal cells (Muscari et al., 2005) and cardiac myoblasts (Tantini et al., 2006). DFMO application resulted in decreasing of both polyamine levels and caspase-3 activity, suggesting a link between polyamine levels and cell apoptosis (Figure 3.7). This reaction benefits morphological transitions in the gill epithelium, because seawater MRCs must undergo apoptosis to reduce the number of seawater MRCs, or they can be replaced by freshwater MRCs (Laurent et al., 2006). Therefore, it is plausible to suggest a link between polyamine levels, cell apoptosis and gill remodeling.

Recent results have shown that increased ornithine decarboxylase activity and polyamine levels at the 6 h and 1 d (Chapter 2) were concomitant with c-myc and c-fos transcription increases in killifish gills (Figure 3.7 panel A and B). This relationship was described in a previous study in rat kidney cells (Tabib and Bachrach, 1994). Although
the relationship between increasing polyamine levels and transcriptional regulation of \( c-fos \) and \( c-myc \) genes in the gill after hypoosmotic challenge exists, the causal relationship between them is unclear. Previous studies have shown that polyamines, including putrescine, spermidine and spermine, can positively regulate \( c-fos \), \( c-ras \), and \( c-myc \) transcription (Bachrach et al., 2001; Li et al., 1998; Liu et al., 2005; Tabib and Bachrach, 1994; Tabib and Bachrach, 1999; Thomas and Thomas, 2003). Moreover, \( c-myc \) and \( c-fos \) mRNA expression was inhibited by DFMO, an inhibitor of ornithine decarboxylase, in carcinoma cells (Celano et al., 1988; Wang et al., 1993) and in cultured cells (Tabib and Bachrach, 1994). These studies suggest that polyamines have the ability to stimulate transcriptional \( c-fos \) and \( c-myc \) genes during environmental challenges (Yuen et al., 2001).

3.4.3 The influence of polyamines on osmotic properties in the opercular epithelium

Most eukaryotic cells have a polyamine transporter system on their cell membrane that facilitates the internalization of exogenous polyamines (Wang et al., 2003). Intracellular polyamines can plug the receptor channel pore, or permeate the ion channel of these receptors. Therefore, the interactions of intracellular polyamines and ion channels have been reported (Williams, 1997). Exogenous polyamines modulate some types of glutamate receptors, such as increase the size of glutamate receptor (Williams, 1997). Overexpression of intracellular polyamine or exogenous addition of polyamines causes the loss of intracellular polyamine homeostasis (Gerner and Meyskens, 2004). Recently, the interactions of intracellular polyamines with different types of ion channels have been reported, including blocking of some types of ion channels and glutamate receptors (Williams, 1997). Polyamines are believed to regulate cell volume during
hypotonic challenges (Watts et al., 1996). Following transfer from 5 ppt to either 0.1 ppt or 0.5 ppt, the plasma osmolality dropped approximately 20% at 1 d and 6 h post transfer, and required approximately 3 d to return to its original level (Figure 3.1). Zadunaisky et al. (2005) suggested a relationship between plasma osmolality and Cl\(^-\) secretion rate in MRCs. In order to test this hypothesis, the opercular epithelium is used as a surrogate of marine fish gill. The opercular epithelium of killifish, a gill-like membrane rich in MRCs, is used to study the mechanism of active Cl\(^-\) secretion in vitro. When the opercular epithelium was subjected to hypotonic solutions on the basolateral side, the \(I_{sc}\) dramatically decreased (Figure 3.5). In this tissue, \(I_{sc}\) is almost entirely derived a Cl\(^-\) secretory current, a distinguishing feature of a marine phenotype (Degnan et al., 1977; Krogh, 1938). \(I_{sc}\) decreased immediately across the opercular membrane upon hypotonic exposure, which could be explained partly by swelling-activated Cl\(^-\) channels on the basolateral membrane (Hoffmann et al., 2002; Hoffmann et al., 2007).

Pre-treatment with a polyamine mixture containing all three polyamines or spermidine alone decreased the magnitude of the hypotonically-induced reduction in \(I_{sc}\) immediately (Figure 3.5 panel A), which indicates that polyamines moderate the inhibition of active Cl\(^-\) secretion in vitro. The paradox is that this effect prevents the reduction in epithelial Cl\(^-\) efflux associated with hypotonically induced cell swelling. Moreover, exhausting polyamine levels by DFMO (Figure 3.4 panel C) attenuated the decrease in Cl\(^-\) transport across the epithelium during hypoosmotic exposure, which indicates that polyamines prevented the reduction in \(I_{sc}\) associated with cell swelling. It is unclear about the mechanism; however, endogenous polyamines are known to block ion channels, such as inward rectifier K\(^+\) channels (Doupnik et al., 1995), intracellular K\(^+\)
and Ca\(^{2+}\) channels (Scott et al., 1993), and Na\(^+\) and Ca\(^{2+}\) fluxes (Bowie and Mayer, 1995; Isa et al., 1995), to control the resting membrane potential and the excitability of cell membranes. Polyamines also moderate Na\(^+/\)K\(^+/2\)Cl\(^-\) co-transporter (NKCC) and cystic fibrosis transmembrane conductance regulator (CFTR), which are involved in mediating cell swelling during hypotonic shock (Hoffmann, 1992; Hoffmann, 2000; Hoffmann et al., 2002; Hoffmann et al., 2007).

The overall conductance, as a rough estimate of ionic permeability, is lower in euryhaline fish acclimated to hypotonic media compared to the level in isotonic media (Figure 3.5 panel B and 3.6 panel B), as shown in previous studies (Marshall et al., 2000; Motais et al., 1969). Spermidine pretreatment prevented both a decrease in membrane conductance (\(G_t\)) (Figure 3.6 panel B) and \(I_{sc}\) (Figure 3.6 panel A). The gill epithelium is more permeable to cations than anions (House and Maetz, 1974), which may explain why Cl\(^-\) transport was inhibited by spermidine over time (Figure 3.6 panel B). Although the mechanism is currently unknown, polyamines, especially spermidine, inhibit the reductions in \(I_{sc}\) and \(G_t\). These findings may help maintain Cl\(^-\) concentration and regulate the amount of seawater-type MRCs in gill epithelia.

Polyamines may facilitate cell apoptosis and cell proliferation with the goal of remodeling the gill, especially in MRCs. The remodeling of MRCs is a rapid physiological adjustment to cope with short bouts of freshwater exposure, and helps euryhaline fish survive in environmental salinity perturbations. However, polyamine accumulation inhibited Cl\(^-\) secretion from MRCs, and resulted in MRC swelling. Further studies are needed to determine the role of polyamines in cell volume regulation and gill remodeling.
CHAPTER 4: THE INFLUENCE OF OSMOTIC STRESS ON TRANSCRIPTION AND TRANSLATION OF ARGINASE, ORNITHINE DECARBOXYLASE, AND POLYAMINE OXIDASE IN TISSUES

4.1 Introduction

Previous studies have shown that hypoosmotic exposure can stimulate polyamine biosynthetic pathways, leading to an increase in putrescine, spermidine, and spermine concentrations in the killifish gill (Chapter 2). With prolonged fresh water exposure, spermidine and spermine concentrations decrease to control levels, whereas the gill putrescine concentration remains elevated (Chapter 2). These data suggest the polyamine biosynthetic pathway is upregulated during acute hypoosmotic transfer and that the polyamine catabolic pathway may become upregulated for late compensatory response of hypoosmotic transfer.

Polyamines are produced in part by the coordinated actions of arginase II (Arg II) and ornithine decarboxylase (Odc) (Pegg and McCann, 1982). Arginase catalyzes a reaction converting arginine to ornithine, a substrate of ornithine decarboxylase (Odc). Odc is a rate-limiting enzyme for the production of polyamines (Pegg and McCann, 1982), catalyzing the reaction converting ornithine to putrescine. This is the first step in the polyamine biosynthetic pathway (Tobias and Kahana, 1993). Putrescine is converted to spermidine and spermine via spermidine synthase and spermine synthase, two enzymes that had low turnover rate and are important sites for polyamine biosynthesis. The polyamine catabolic pathway consists of reactions involving the acetylation and oxidation of larger polyamines into smaller polyamines. It primarily includes the acetylation of spermidine or spermine by spermidine/spermine -N1-acetyltransferase (SSAT) and the oxidation of N1-acetyl spermidine and N1-acetyl spermine to putrescine and spermidine, respectively by polyamine oxidase (Pao) (Seiler, 2004). Though both SSAT and Pao are
key enzymes for polyamine catabolism, Pao is the terminal oxidase that regulates the ratio of spermidine to spermine (Adibhatla et al., 2002).

The key enzymes for polyamine metabolism have wide tissue distribution. Previous studies have shown that Arg II is widely expressed in many extrahepatic tissues, such as intestine and kidney in rainbow trout (Wright et al., 2004). Moreover, Pao activity has been studied in the liver, kidney, uterus, brain, lung, and skeletal muscles of rats (Pavlov et al., 1991), humans (Suzuki et al., 1984) and catfish (Kumazawa and Suzuki, 1987). The ubiquity of key enzymes for polyamine metabolism supports the importance of polyamines across tissue types and organisms. It is necessary to know if Arg, Odc and Pao are present in the tissues and organs of killifish. Only limited information on the tissue distribution is available at present; therefore, the influence of hypoosmotic challenges on the enzymatic activities of Arg, Odc and Pao was investigated in multiple tissues including gill and intestine (osmoregulatory organs), liver, heart and muscle (energetic metabolism organs), spleen (immune system organ) and testes (reproductive organ).

4.2 Materials and methods

4.2.1 Experimental animals

Adult F. grandis were collected from a local hatchery (Gulf Coast Minnows, Thibodaux, LA, USA) and maintained in a 330-L aquarium with a single recirculation system containing mechanical and biological filtration and UV-sterilization. Salinity was maintained at 5 ± 0.1 ppt water, which was prepared by diluting Instant Ocean® (Blacksburg, VA, USA) with reverse osmosis water. Temperature was kept at 23 ± 1 ºC, and salinity and temperature were monitored daily using a water quality meter (YSI,
Yellow Spring, OH, USA). Partial replacement of system water was performed at least twice a week to maintain ammonia and nitrite below the detection levels of commercial kits (Aquarium Pharmaceuticals, Chalfont, PA, USA). Dissolved oxygen was kept near air-saturation using a low-pressure aerator. A 12 h light:12 h dark cycle was provided by fluorescent lighting. Fish were fed a commercial pellet (Cargill, Franklin, LA, USA) twice daily at 2% body weight per day throughout the study.

4.2.2 Experimental design

Sixty adult fish weighing between 7.8 and 11.5 g were acclimated to 5 ppt water before being randomly divided into two groups. Following acclimation, fish were transferred to aquaria containing either 5 ppt or 0.1 ppt water for one week and fed twice daily until 12 h before sampling. Following exposure, fish were killed by caudal severance and testes, gills, intestine, liver, heart, cranially-located epaxial muscle, and spleen were collected from each fish. The intestinal tract from the end of the pyloric sphincter to the anus was sampled as the whole intestine. Tissues (N=6 for each salinity) were immediately stored in at least 10-times volume of RNALater (Invitrogen, Carlsbad, CA, USA), stored at 4 °C overnight, and transferred to a -20 °C freezer until total mRNA extraction. Distribution of arg II and odc mRNA levels in tissues of adult *F. grandis* acclimated to 5 ppt water was analyzed using RT-PCR. PCR products were run on a 2% agarose gel to confirm the presence of a single product. All cDNA samples were subsequently used in qPCR to assess the relative tissue distribution of arg II and odc mRNA in tissues, as described in Section 4.2.3. Tissues were flash frozen in liquid nitrogen and stored at -80 °C until the activities of arginase, Odc, and Pao were measured, as described in Sections 4.2.4, 4.2.5, and 4.2.6, respectively.
4.2.3 arg II and Odc mRNA levels

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for the extraction of total RNA from tissues according to the manufacturer’s instructions. Expressed sequence tags (EST) for arg II (AB290198.1) and odc (CN977303.1) from F. heteroclitus were obtained using the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/) and used to derive forward and reverse primers for quantitative PCR (Integrated DNA Technologies Inc., Coralville, IA, USA). qPCR analysis was performed for the target genes, arg II and odc, and 18S ribosomal RNA (18s rRNA) was used as the reference gene. Analysis was conducted using a commercial kit (SYBR Green PCR Core Reagent, Applied Biosystems, Carlsbad, CA, USA) with an ABIPRISM 7000 Sequence Detection System (Carlsbad, CA, USA). Reagents included 5 µL cDNA (0.1 µg), 2 µL forward primers (10 nM), 2 µL reverse primers (10 nM), 5 µL SYBR Green reagent, and 6 µL Ultrapure water (Invitrogen, Grand Island, NY, USA) in a total volume of 20 µL. Samples were cycled 40 times at 95°C for 15 s and 60°C for 1 min. Relative changes in mRNA levels were made using the 2^{-ΔΔCt} method (Fleige et al., 2006).

4.2.4 Total Arginine activity in multiple tissues

Total arginase activity was measured as described previously (Mommsen et al., 1983). Briefly, tissues were homogenized in HEPES buffer (50 mM, pH=7.5) (V:V for 1:5) and centrifuged at 12,000 g for 4 min at 4°C. Supernatants were incubated in 50 mM MnCl₂ for 10 min, and the pre-incubated supernatant was mixed with reaction reagent (250 mM L-arginine, 1 mM MnCl₂, and 50 mM HEPES (pH=8.0). After 30 min, 25 µL 70% perchloric acid was added to the reaction mix and then neutralized with
KHCO₃. The enzymatic reaction was performed at room temperature and the reaction mix was centrifuged at 12,000 g for 4 min at 4 °C. The supernatant was analyzed for total urea using a QuantiChrom urea assay kit (Bioassay System, Hayward, CA, USA), and total arginase activity was expressed in µmol urea/h/ g tissue.

4.2.5 Decarboxylase activity in multiple tissues

The activity of ornithine decarboxylase did not differ in fresh tissue or in tissues flash frozen at the time of collection in liquid nitrogen and stored at -80°C (data not shown). Therefore, all Odc activity assays were performed on frozen gill samples, which were thawed on ice and homogenized in reaction buffer (50 mM K₂HPO₄ buffer (pH 7.5), 5 mM dithiothreitol, 0.2 mM EDTA, 0.05 mM pyridoxal 5-phosphate). Samples were centrifuged at 20,000 g for 20 min at 0 °C. The supernatant was added to a 16 mm x 100 mm borosilicate tube with an airtight seal formed with a double seal rubber stopper (Kimble Chase Kontes, K-882310) and infiltrated by a plastic centerwell (Kimble Chase Kontes, K-882320). The centerwell contained 1.3 cm² of Whatman #1 filter paper saturated with 133 µL 40% KOH. A 0.5 µCi mixture of DL-[¹⁴C] ornithine hydrochloride (52 mCi/mmol, Moravek Biochemicals, Brea, CA, USA) and cold L-ornithine (Sigma, St. Louis, MO, USA) were added anaerobically to the supernatant. The ornithine concentration used in the assay mixture (1.2 mM) was the same as used in a previous study (Watts et al., 1996). The reaction mixture was kept in a water bath at 25 °C for 90 min, and 5% trichloroacetic acid was added to stop the reaction. Radiolabeled ¹⁴CO₂ was collected overnight on filter paper saturated with 40% KOH. The filter paper was placed in 5 mL Ultima Gold cocktail (Perkin-Elmer, Waltham, MS, USA), and total ¹⁴CO₂ was measured using a Beckman LS 6500 Liquid Scintillation Counter (GMI Inc.)
Ramsey, Minnesota, USA) as described by Watts (Watts et al., 1996). Internal standardization demonstrated both constant and negligible $^{14}$C quench, so no quench correction was applied to the data analysis. Odc activity was expressed as nmol CO$_2$ produced/h/g tissue.

4.2.6 Testing polyamine oxidase (Pao) activity in different tissues

Tissues were homogenized in a 15-fold volume of MilliQ water with homogenizer (PRO 200, Lab depot Inc. Dawsonville, GA, USA). The crude homogenate was used as a polyamine oxidase source. The assay method for Pao was modified from the protocol description by Suzuki (Suzuki et al., 1984), in which Pao catalyzed homovanillic acid to a highly fluorescent compound. The fluorescence intensity was measured with excitation at 355 nm and emission at 460 nm using a VICTOR™ X3 multi-label plate reader (Perkin Elmer, Santa Clara, CA, USA). The assay mixture without substrate ($N^1$-acetylspermine trihydrochloride) was incubated as a blank control, and 2.2 nmol H$_2$O$_2$ was added instead of homovanillic acid as an internal standard.

4.2.7 Statistical analyses

One-way ANOVA and the Tukey post hoc test were performed using SAS (v. 9.3, SAS Institute Inc., Cary, NC, USA) to test for significant differences within tissues in both the 5 ppt and 0.1 ppt treatments. Statistical differences were considered significant at $P$-values $\leq$0.05.

4.3 Results

4.3.1 Tissue distribution of arg II and odc

The mRNA levels of arg II and odc were measured in the gills, intestine, testes, heart, muscle, liver, and spleen relative to 18s rRNA. All mRNA levels were normalized
to mRNA level of the gill in 5 ppt-acclimated fish. PCR primers used to amplify arg II (Figure 4.1 panel A) and odc (Figure 4.2 panel A) produced single amplicons in all tissues of adult male killifish, as demonstrated by 2% agarose gel electrophoresis of PCR products. Quantitative PCR was subsequently used to assess the relative tissue distribution of arg II (Figure 4.1 panel B) and odc (Figure 4.2 panel B) following 7 d exposure of F. grandis to either 5 ppt or 0.1 ppt water. ArgII mRNA transcript was highly expressed in the gills, regardless of salinity (P<0.05), compared to all other tissues (Figure 4.1 panel B). Moreover, arg II mRNA levels increased significantly only in the gills (P=0.0459), liver (P=0.0099) and spleen (P=0.0106) of fish acclimated to 0.1 ppt water compared to the 5 ppt controls (Figure 4.1 panel B).

Although odc mRNA was most highly expressed in the testes of male killifish, there were no significant differences in mRNA levels between the testes and gills in 5 ppt or 0.1 ppt-acclimated fish (Figure 4.2 panel B). In contrast, odc mRNA levels were much higher in the gills than in other tissues, except for the testes in both 5 ppt and 0.1 ppt-treated fish. Low odc mRNA levels were observed in the spleen, muscle, and heart. Odc mRNA levels were not significantly different from one another in all observed tissues between 5 ppt and 0.1 ppt–acclimated fish at 7 d post-transfer (Figure 4.2 panel B).

4.3.2 Arginase (Arg) and ornithine decarboxylase activities in tissues of F. grandis

After determining the mRNA levels of arg II and odc genes, Arg and Odc activities were analyzed. Despite the fact that the gills had the highest abundance of arg II mRNA transcripts of all the tissues analyzed, the liver had a significantly higher level of Arg activity (P<0.0001 for both salinities). The testes, intestine, muscle, and spleen had a similar level of Arg activity (P>0.05 for both salinities), whereas the heart had a
Figure 4.1: (A) Semi-quantitative assessment of arg II mRNA in the tissues of *F. grandis* acclimated to 5 ppt water using RT-PCR analysis. SM represents 50 bp size marker, and G, T, I, L, H, M, and S represent the gills, testes, intestine, liver, heart, muscle, and spleen, respectively. (B) Relative arg II mRNA levels in tissues of *F. grandis* acclimated to 5 ppt or 0.1 ppt water for 7 d (N = 6 per salinity). 18S ribosomal RNA was used as a reference gene, and arg II mRNA levels were normalized to the mRNA level of the gills (5ppt-acclimated fish) and assigned a value of 1. Lowercase letters indicate a significant difference (*P* ≤ 0.05) in comparison to gills at 5 ppt for all tissues; uppercase letters indicate a significant difference (*P* ≤ 0.05) in comparison to gills in 0.1 ppt exposed fish; asterisks indicate a significant difference (*P* ≤ 0.05) in comparison to the 5 ppt control.
Figure 4.2: (A) Semi-quantitative assessment of odc mRNA in tissues of *F. grandis* acclimated to 5 ppt water using RT-PCR analysis. SM represents 50 bp size marker; G, T, I, L, H, M, and S represent the gills, testes, intestine, liver, heart, muscle and spleen, respectively. (B) Relative odc mRNA levels in tissues of *F. grandis* acclimated to 5 ppt or 0.1 ppt water for 7 d (N = 6 per salinity). 18S ribosomal RNA was used as a reference gene, and odc mRNA levels were normalized to the mRNA level of the gill (5 ppt-acclimated fish) and assigned a value of 1. Lowercase letters indicate a significant difference (*P* ≤ 0.05) in comparison to the gill at 5 ppt for all tissues; uppercase letters indicate a significant difference (*P* ≤ 0.05) in comparison to the gill in 0.1 ppt water exposed fish; asterisks indicate a significant difference (*P* ≤ 0.05) in comparison to the 5 ppt control.
significantly lower level of Arg activity \((P<0.0001\) for both salinities) compared to the gills (Figure 4.3). Hypoosmotic exposure only increased Arg activity in the gills \((P=0.0184)\) compared to the 5 ppt control, but did not affect enzymatic activity in other tissues (Figure 4.3).

Figure 4.3: Arginase activity in the tissues of adult *F. grandis* acclimated to 5 ppt and 0.1 ppt water for 7 d. Data are represented as mean values ± SEM \((N = 6)\) in µmol urea produced \(/h/g\) tissue. Lowercase letters indicate a significant difference in levels of different tissues \((P \leq 0.05)\) in comparison to the level of gills in 5 ppt treated fish; uppercase letters indicate a significant difference in levels of different tissues \((P \leq 0.05)\) in comparison to the gill in 0.1 ppt treated fish; asterisks indicate a significant difference \((P \leq 0.05)\) in the tissues from the 0.1 ppt exposed fish in comparison to those from the 5 ppt-acclimated fish.

There was no significant difference in Odc activity between all tissues measured in 5 ppt-acclimated fish (Figure 4.4). After transfer to 0.1 ppt water, the gills had higher Odc activity than all other tissues except the testes (Figure 4.4). Hypoosmotic transfer increased Odc activity in the gills \((P=0.0044)\), but did not affect Odc activity in other tissues (Figure 4.4).
Figure 4.4: Ornithine decarboxylase activity in tissues of *F. grandis* acclimated to 5 ppt and 0.1 ppt water for 7 d. Data are represented as mean values ± SEM (N = 6) and expressed as nmol CO₂ produced/h/g tissue. Lowercase letters indicate a significant difference in levels of different tissues (*P* ≤ 0.05) in comparison to the level of gill in 5 ppt treated fish; uppercase letters indicate a significant difference in levels of different tissues (*P* ≤ 0.05) in comparison to the gill in 0.1 ppt treated fish; asterisks indicate a significant difference (*P* ≤ 0.05) in the tissues from the 0.1 ppt exposed fish in comparison to those from the 5 ppt-acclimated fish.

4.3.3 Pao activity in multiple tissues

To assess the extent of polyamine catabolism, Pao activity in multiple tissues of *F. grandis* acclimated to 5 ppt and 0.1 ppt water was measured. After 7 d acclimation to 5 ppt and 0.1 ppt water, the intestine, liver, and gills had higher Pao activity than that in other tissues, as shown in Figure 4.5. Hypoosmotic exposure increased Pao activity in the gills (*P*=0.0094), but did not affect the Pao activity of other tissues, compared to tissues from 5 ppt-acclimated fish (Figure 4.5). The gill had the highest Pao activity compared to all other tissues during hypoosmotic exposure.
Figure 4.5: Pao activity in tissues of *F. grandis* after 7 d of hypoosmotic exposure. Data are represented by mean ± SEM (N=6) and expressed as nmol/g tissue /30 min. Lowercase letters indicate a significant difference in levels of different tissues (P ≤ 0.05) in comparison to the level of gill in 5 ppt treated fish; uppercase letters indicate a significant difference in levels of different tissues (P ≤ 0.05) in comparison to the level of gill in 0.1 ppt treated fish; asterisks indicate a significant difference (P ≤ 0.05) in the tissues from the 0.1 ppt water exposed fish in comparison to those from the 5 ppt-acclimated fish.

4.4. Discussion

4.4.1 The enzymatic activity of arginase, ornithine decarboxylase and Pao in the gills

This study suggests that changes in enzymatic activity associated with polyamine metabolism in gills may be an important response of euryhaline fish to acute hypoosmotic exposure. The fish gill is a multipurpose organ that plays dominant roles in osmotic and ionic regulation, acid-base regulation, gas exchange, and excretion of nitrogenous wastes (Evans *et al.*, 2005; Wood and LeMoigne, 1991; Wood and Marshall, 1994). In *F. grandis*, hypoosmotic challenges increased the enzymatic activities of Arg, Odc and Pao only in the gills (Figures 4.3, 4.4 and 4.5). This indicates that although all
tissues had measurable levels of enzymatic activities associated with polyamine metabolism, only the gill had enzyme activities affected by hypoosmotic challenge. The intracellular concentrations of putrescine, spermidine, and spermine are strictly regulated by multiple biochemical pathways involving Arg, Odc, and Pao, suggesting polyamines play an important role in various physiological functions in plants and animals (Galston, 1983; O’Brien et al., 1975; Sekowska et al., 1998). Putrescine participates in modulation of gene transcription, signal transduction, membrane stability and receptor-ligand interactions; spermidine participates in modulation of gene translation and chromatin structure; and spermine is involved in modulation of mRNA/DNA stabilization, cell growth and proliferation (Pegg and McCann, 1982; Ray et al. 1999; Tabor and Tabor, 1984). This study suggests that hypoosmotic exposure stimulates polyamine biosynthetic pathways, resulting in an increase in putrescine, spermidine and spermine concentrations in the gill (Chapter 2). Highly cationic polyamines such as spermidine and spermine mediate early phase compensatory responses in the euryhaline killifish when faced with osmotic challenges. Although gill spermidine and spermine increased transiently in killifish with freshwater exposure, putrescine remained elevated over time, suggesting a possible role of this polyamine in acclimation of killifish to fresh water. Moreover, spermidine, and spermine concentrations increased in the gills of Callinectes danae during hyperosmotic exposure (Silva et al., 2008). Elevated putrescine and spermidine concentrations were also present in the gills of Callinectes sapidus after hyperosmotic exposure (Lovett and Watts, 1995). In contrast, the putrescine concentration of Artemia nauplii decreased with an increase of environmental salinity (Watts and Good, 1994). Although there was no effect on spermidine and spermine
concentration, Odc activity and putrescine concentration increased in brine shrimp during hypoosmotic exposure (Watts et al., 1996). Presumably, salinity challenges stimulated perturbation of polyamine homeostasis in order to mediate a series of biological functions, such as stimulating cell apoptosis (Laurent et al., 2006). In this study, hypoosmotic exposure resulted in upregulation of key enzymes of polyamine metabolism, although additional studies are needed to determine the physiological effects of individual polyamines to hypoosmotic exposure. Surprisingly, the increase in Odc activity during hypoosmotic exposure was not the result of an increase in odc mRNA level at 7 d hypoosmotic exposure (Figures 4.2 and 4.4). Actually, Odc activity is regulated by translational as well as post-translational mechanisms (Lovkvist et al., 2001). This phenomenon can be explained mainly by post-transcriptional regulation. Previous studies have also shown that hypoosmotic challenge increased Odc activity without any detectable change in Odc mRNA level in L-1210 cell line (Poulin and Pegg, 1989). As such, the induction of Odc activity can be explained partly by an increased efficiency of Odc mRNA translation.

4.4.2 The enzymatic activity of arginase, ornithine decarboxylase and Pao in testes, intestine, liver, heart, muscle and spleen

Hypoosmotic exposure did not affect the activities of Arg, Odc and Pao in testes, intestine, liver, heart, muscle and spleen. Among those tissues, liver had the highest arginase activity in F. grandis (Figure 4.3) in both salinities, in accordance with earlier results of arginase activity in other fish species (Cvancara, 1969). However, the liver had a low expression of arg II mRNA (Figure 4.1 panel B). Morris (2004) suggests that Arg I, not Arg II, is the primary arginase protein in the liver. In addition, studies have shown that arg I mRNA levels are higher than the levels of arg II in the liver of adult rainbow
trout (Wright et al., 2004), humans (Morris et al., 1997), and Xenopus tadpoles (Patterton and Shi, 1994). Thus, Arg I mainly contributed to liver arginase activity. There is no evidence of Arg I in Fundulus, and most teleost fish are obligate ammonioteles lacking a functional OUC beyond embryogenesis; thus, Arg II is the major functional arginase in them to produce ornithine (Wright et al., 2004; Wu et al., 1998). Moreover, as a substreate of arginase, arginine is a precursor of polyamines and various amino acids, such as proline and glutamate. Arginase activity in fish is found in high abundance in tissues with high metabolic activity, such as the liver, because these tissues play an important role in amino acid metabolism in fish (Ketola, 1982).

Another key enzyme for polyamine biosynthesis is Odc. In this study, Odc activity was higher in the testes compared to all other tissues, excluding the gills in 0.1-ppt acclimated killifish (Figure 4.4). Odc is the rate-limiting enzyme for polyamine biosynthesis, and polyamines, namely putrescine, spermidine and spermine, are present in and synthesized by germ cells, Sertoli, and Leydig cells of the testis in the rat (Lefèvre et al., 2011). Moreover, this study shows that the liver and intestine had high Pao activity in F. grandis tissues (Figure 4.5). The intestine had higher Pao activity for both salinities at 7 d, which is consistent with the high Pao activity observed in the intestine of catfish (Kumazawa and Suzuki, 1987). The intestine is responsible for water and food absorption, and this action can compensate for water loss from kidney (Evans et al., 2005). Thus, intestine is an important organ for osmotic regulation. Besides the intestine, liver has high Pao activity because it functions in lipid storage and works as an enzymatic pool in other species (de la Torre et al., 1999).
In summary, biosynthetic and catabolic pathways tightly regulate polyamine levels by multiple tissues. All polyamines components played a role in dealing with hypoosmotic challenge; however, spermidine and spermine levels dropped after 1 d. Gill putrescine stored at early and later compensatory response to hypoosmotic challenge. High Arg, Odc and Pao activity in the gills, intestine and liver are presumably involved in polyamine homeostasis in the killifish. The ratio of putrescine level over the sum concentration of spermidine and spermine reached its maximum at 7 d post-transfer, suggesting more putrescine accumulated in the gill at later compensatory response to hypoosmotic challenge (Chapter 2). This study indicates putrescine may play a role in the recovery *F. grandis* from hypoosmotic exposure. Gill remodelling is a strategy for killifish survival in hypoosmotic exposure, although a link between putrescine and gill remodelling needs clarification. Therefore, the role of putrescine in gill remodelling during hypoosmotic exposure should be further studied.
CHAPTER 5: CONCLUSION

Polyamines are a family of low molecular weight organic cations, and they have been implicated in diverse physiological processes. My research to date has described a possible role of polyamines in the acute response of the gulf killifish, *Fundulus grandis*, to hypoosmotic exposure. The purpose of this study was to describe the transcription and enzymatic activities of key enzymes for polyamine metabolism, to assess polyamine levels during this process, and to assess the putative roles of polyamines in the gills of *F. grandis* during hypoosmotic challenges. Also, the influence of the irreversible inhibitor ornithine decarboxylase (Odc) by alpha-DL-difluoromethylornithine (DFMO) was used to gain insight to the influence of impairment of polyamine biosynthesis on the physiological response of killfish to freshwater exposure. The current study sought to answer the following questions: a) Do osmotic challenges influence the synthetic and catabolic pathways that regulate gill polyamine concentrations? b) Do polyamines play a role in gill epithelial remodeling during hypoosmotic exposure? c) Does polyamine accumulation play a role in Cl\(^-\) secretion associated with cell swelling *in vitro* during hypoosmotic exposure? and d) Does polyamine metabolism occur in multiple tissues during hypoosmotic challenges in killfish?

Major empirical findings from my first study showed that a large increase in mRNA levels and the enzymatic activities of Arg and Odc in the gills occurred in response to exposure of killfish to low environmental salinity (Figures 2.1 and 2.2). One of the most significant results to emerge from this study was that *odc* mRNA levels increased exponentially as environmental salinity decreased at all observed time points (data not shown). Moreover, the levels of spermidine and spermine were significantly
elevated in the gills at only 1 d exposure to 0.1 ppt, whereas the putrescine level was significantly elevated by this treatment at 1 d and beyond (Figure 2.3). The ratio of putrescine level over the sum of spermidine and spermine levels increased progressively following transfer to 0.1 ppt water (Table 2.2). This change in this ratio is associated with a progressive reduction in the concentrations of spermidine and spermine, yet a sustained elevation in the concentration of putrescine. This change in the ratio also suggests that both spermine and spermidine mediated the acute compensatory response of hypoosmotic challenges in the killifish; whereas, putrescine play an important role in the prolonged compensatory response of hypoosmotic shock in the euryhaline gulf killifish. Gill putrescine reached its maximum concentration at 7 d post-transfer to hypoosmotic environment, concomitant with an increase in polyamine oxidase activity (Figure 4.5), suggesting that polyamine catabolism was upregulated following hypoosmotic exposure (0.1 ppt). The polyamine catabolism was not measured at early time points; therefore, it cannot conclude how polyamine catabolism changed. However, polyamine levels should be kept in a homeostasis state, and polyamine catabolism has a high possibility to upregulated initially. Polyamine catabolism is a back-conversion pathway, converting highly aminated polyamines, such as spermine and spermidine, back to putrescine. These results showed that osmotic challenges affected gill polyamine levels by regulating both polyamine biosynthesis and catabolism. Putrescine, spermidine and spermine may play a role in different temporal phases after freshwater exposure.

This study showed that the efficacy of DFMO on Odc activity was dependent on exposure salinity. Odc activity and polyamine concentrations were significantly reduced by DFMO after 0.1 ppt transfer; however, DFMO had no effect on endogenous Odc.
activity in fish gills in the absence of hypoosmotic exposure (Table 2.3; Figures 2.4 and 2.5). Surprisingly, gills still exhibited a marked reduction in gill polyamine concentrations with DFMO despite the inability to inhibit the enzyme. This result may be explained by the polyamine-mediated feedback of Odc activity. Polyamines exerted an inhibitory effect on Odc synthesis, and they also stimulated Odc degradation by inducing antizyme synthesis. Another explanation may be the existence of DFMO-insensitive Odc. A large component of total Odc activity in the killifish gill is DFMO-insensitive Odc, especially the endogenous sources not stimulated by hypoosmotic exposure.

Another significant finding to emerge from this study was a strong correlation between the increase of arg II and odc mRNA levels and the morphological transition in MRCs during hypoosmotic exposure (Figures 3.2 and 3.3). This strongly suggests that upregulation in polyamine biosynthesis may either mediate some of the phenotypic changes to the gill epithelium or that it is a consequence of the cellular swelling and/or damage during the early phases of freshwater transfer. Hypoosmotic challenges induce a morphological transition from seawater-type to freshwater-type MRCs in the gill epithelium (Figure 3.2). Concurrent with the onset of morphological changes to the gill, FW exposure elicited increases in the mRNA levels of c-fos, c-myc oncogenes, and caspase-3 activity (Figure 3.7). These results suggested that polyamine-mediated cell proliferation and apoptosis may be involved in gill remodeling after freshwater exposure. Moreover, DFMO application reduced not only caspase-3 activity, but also the levels of gill putrescine, spermidine and spermine after hypoosmotic exposure, indicating a relationship between polyamines and cell apoptosis in the low environmental salinity.
The next goal of this thesis was to assess a possible association between gill polyamine accumulation and cell volume disturbances or regulated recovery from cell volume disturbance. This work was conducted by supplementing isolated opercular epithelia exposed to hypotonic conditions with putrescine, spermidine, or spermine, either singly or as a polyamine mixture. Opercular epithelium was used for this study because it shares many similar properties to the gills, including the presence of MRCs, the existence of a transcellular Cl\(^-\) secretory mechanism similar to that found in MRCs of marine fish gill, and has the capacity to undergo regulatory volume decrease in the face of hypotonic swelling. As a proxy of cell swelling, short circuit current (\(I_{sc}\)) was used to reflect Cl\(^-\) secretion across the epithelium. A hypotonic shock was applied on the basolateral side of opercular epithelia. A decrease of 60 mOsM/Kg reduced \(I_{sc}\) by approximately 60\% and hypotonic inhibition of \(I_{sc}\) was accompanied by reduction in membrane conductance (\(G_t\)). Administration of spermidine to the basolateral side of the opercular epithelium prevented a decrease in \(G_t\) and \(I_s\) across the epithelium induced by hypotonic exposure (Figures 3.4, 3.5 and 3.6). Thus, polyamine accumulation may aggravate the cell swelling by inhibiting Cl\(^-\) secretion \textit{in vitro} after hypotonic exposure. Finally, this study shows that the transcriptional and enzymatic activities of Arg, Odc and Pao were upregulated in the gills, intestine and liver during acute freshwater exposure, suggesting that polyamine levels are regulated on the whole-animal level in killifish (Figures 4.1, 4.2, 4.3, 4.4, and 4.5). Intestine is another important osmoregulatory organ, and it gains extra water through food ingestion to compensate for renal elimination after transfer to fresh water. Low activities for Arg and Odc, and a high activity of Pao suggest the intestine plays an important role in polyamine metabolism. Pao participates
in the regulation of putrescine concentration during extended freshwater exposure, supporting a possible role of putrescine in maintenance of the FW gill phenotype.

These studies have offered a comprehensive perspective on the regulation of polyamine metabolism in killifish. Increases in putrescine, spermidine and spermine during osmotic challenges were observed in the killifish, and this pattern facilitated fish to acclimate to fresh water by gill remodeling. Moreover, long-term exposure of killifish to FW leads to the persistent elevation of gill putrescine concentration, but not in the more highly aminated spermidine and spermine. This result suggested a relationship between putrescine concentration in the gill and gill remodeling in the prolonged compensatory response to hypoosmotic challenge. However, polyamine accumulation may aggreavate cell swelling in MRCs by preventing Cl\(^-\) secretion in the face of hypotonic shock, which may be related to the functions of Na\(^+\)/K\(^+\)/2Cl\(^-\) co-transporter (NKCC) and cystic fibrosis transmembrane conductance regulator (CFTR) during hypotonic shock (Hoffmann, 1992; Hoffmann, 2000), as previous studies have shown. Therefore, it is premature to conclude whether polyamines are protective or cytotoxic to cells during hypoosmotic challenges, although the regulation aimed at maintenance of gill putrescene during FW acclimation supports an important role of this molecule.

Future studies should establish a direct linkage between \textit{arg II} and \textit{odc} mRNA expression and polyamine concentration in the gill. Second, a direct relationship between gill polyamine levels and cell apoptosis should be establashed. Third, although the study showed a correlation between polyamine and \textit{c-fos} and \textit{c-myc} transcription, a causal link between them needs to be clarified during hypoosmoitoic exposure. Forth, DFMO application prevented a decrease in plasma Na\(^+\) and Cl\(^-\) concentrations in the gill after
hypoosmoitic challenge, but clearly understand the causal relationship between polyamines and plasma osmolality needs further work.
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