THE LIMITS OF OSMOREGULATION: STRATEGIES FOR TOLERANCE AND ACCLIMATION OF 'CALIFORNIA' MOZAMBIQUE TILAPIA (OREOCHROMIS MOSSAMBICUS X O. UROLEPIS HORMORUM) TO CONDITIONS OF THE SALTON SEA

By

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ABSTRACT

The Salton Sea is a large inland lake in southeastern California with salinity currently near 44 g/l that is increasing at a rate of 0.3 g/l annually. Along with salinity, large fluctuations in temperature and dissolved oxygen levels combine to make a very challenging environment that may be responsible for dramatic losses to the Salton Sea’s once robust fishery. The dominant species within the fishery is a Mozambique tilapia hybrid (Oreochromis mossambicus x O. urolepis hornorum), which is well known both for euryhalinity and tolerance of extremely high salinities; as such, it provides a unique model for tolerance to hypersaline conditions as well as the interactions of multiple stressors such as those within the Salton Sea. In part one I describe two responses by tilapia to salinities greater than seawater at 25°C. When transfers were conducted below 60 g/l salinity, tilapia maintained osmotic balance without increasing drinking rate, mitochondrial-rich cell (MRC) turnover, or branchial Na⁺, K⁺-ATPase (NKA) activity. With additional increase above 60 g/l, these variables increased in similar fashion to that which has been described in other teleosts during acclimation to elevated salinity. These acclimation responses were defined as response I or response II, with a transition point between the two at 60 g/l. Tilapia exhibiting response I had a reduced whole animal oxygen consumption rate, as well as, liver and brain ATPase activity in proportion to salinity. In part two, I describe how changes in temperature affect the salinity tolerance of this species. Variation in temperature from 25°C to 15 or 35°C resulted in increased plasma osmolality and/or mortality, indicating a combined temperature/salinity stress is more challenging than salinity alone. Using tissue microarrays and laser scanning
microscopy, I show that tilapia attempted to respond to the loss of osmotic balance in cold temperatures with MRC hypertrophy and enhanced NKA capacity.

KEYWORDS: Osmoregulation, ion regulation, tilapia, *Oreochromis mossambicus*, Salton Sea, hypersalinity, tissue microarray, osmorespiratory compromise.
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<td>AC</td>
<td>Accessory Cell</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>CC</td>
<td>Chloride Cell</td>
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<tr>
<td>FW</td>
<td>Fresh Water</td>
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<td>g/l</td>
<td>grams per liter</td>
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<tr>
<td>Hb</td>
<td>Hemoglobin</td>
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<td>Hct</td>
<td>Hematocrit</td>
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<tr>
<td>LSC</td>
<td>Laser Scanning Cytometry</td>
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<td>MCHC</td>
<td>Mean Cell Hemoglobin</td>
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<td>mM</td>
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CO-AUTHORSHIP STATEMENT

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Chapter 4 was published as Sardella, B. A., V. Matey, J. Cooper, R. J. Gonzalez, and C. J. Brauner in the Journal of Experimental Biology 207:1399-1414 (2004). I conducted all salinity transfers, tissue collections, and analysis of plasma osmolality, [Na⁺] and [Cl⁻], as well as blood variables and muscle water content. Victoria Matey prepared all electron micrographs and conducted cell counts. Jill Cooper conducted the Na⁺, K⁺-ATPase activity assays. Oxygen consumption rates were measured by Richard Gonzalez. Data compilation, analysis, and presentation were done by me, as was the preparation of the manuscript.

Chapter 6 was submitted by Sardella, B. A., D. Kültz, J. J. Cech Jr., and C. J. Brauner to the Journal of Experimental Biology as of September, 2006. All experiments, data collection, analysis, and manuscript preparation were conducted by me.
CHAPTER 1: INTRODUCTION AND OVERVIEW

One of the harshest yet least studied environments is the desert saline lake, where temperature and salinity stresses place physiological challenges on fishes. This thesis focuses on the Salton Sea in southern California, where high salinity (~44 g/l) and temperature fluctuations (12-37°C) interact to provide a very challenging environment in which wide-scale fish kills are common (Riedel, 2002). One species that currently inhabits the Salton Sea is a Mozambique tilapia hybrid that has been referred to as the California Mozambique tilapia (*Oreochromis mossambicus* x *O. urolepis hornorum*), a euryhaline species that has been shown to have a high tolerance for hypersalinity (salinities exceeding that of seawater). Using the Salton Sea as an ecological framework for laboratory experiments involving this tilapia hybrid, I will 1) characterize the physiological responses to hypersalinity, 2) investigate the mechanisms behind their previously documented tolerance for extreme salinities, and 3) investigate the interaction of temperature and salinity on osmoregulatory capacity, in order to test the hypothesis that this interaction is a potential cause for the fish kills that occur in the Salton Sea. The remainder of the introduction will provide background information on saline lakes, as well as osmoregulatory mechanisms used by fishes, the effects of elevated salinity on the metabolic rate of fishes, and the effects of temperature/salinity interaction on fish osmoregulation.
**Saline Lakes and the Salton Sea**

Saline lakes are found on every continent outside of Antarctica, comprising a total volume of approximately 104,000 km$^3$ (Williams, 1996; Fig 1.1). Although cosmopolitan, they tend to have similar features with respect to their regional distribution and climate. Saline lakes are most often found in sub-humid to semi-arid desert climates, occasionally existing in arid climates, in spite of low precipitation (Hammer, 1986; Williams, 1996). The high salinities of these lakes are usually maintained by a similar mechanism; a variable inflow coupled with little to no outflow, and a high rate of evaporation. Geographically, they are typically, but not always, found in endorheic drainage basins.

While limnological data is commonly available for saline lakes, information regarding fish species that inhabit them can be difficult to obtain. Saline lakes around the world often have dense populations of algae, bacteria, and invertebrates, but fish are not as common. Hammer (1986) suggested that saline lakes are capable of supporting more fish species than is presently observed, but due to limited dispersal there is a lack of species diversity. Fish can establish populations only in permanent saline lakes and do not benefit from dispersal mechanisms such as birds or winds as invertebrates do. Despite this, fish inhabit some saline lakes, but their occurrence is more often due to introduction by humans in order to establish new fisheries or supplement an existing one (Hammer, 1986).
In North America, saline lakes of several types exist throughout the western part of the continent; many of these lakes are located in desert rain shadows of the Great Basin, Mojave, Sonoran, or Chihuahuan deserts. The Salton Sea is a 980 km$^2$ saline lake that formed in 1905-06 when the Colorado River flooded the Imperial and Coachella Valleys of southeastern California (Fig. 1.2). The Salton Sea is a permanent saline lake in a very arid region. Due to inflow of nutrient and salt-rich water, and a very high rate of evaporation, the salinity of the Salton Sea has increased from levels normally present in freshwater at the time of its formation, to 44 g/l presently, and continues to increase at a rate of 0.3 g/l/year (Watts et al., 2001). The ionic composition is not identical to seawater, as Salton Sea water has twice the calcium concentration and three times the sulphate concentration of seawater concentrated to 44 g/l.
The Salton Sea has a substantial fish population consisting of both native and introduced species. The recreational fishery consists of four species, three of which were introduced from the Gulf of California; orange mouth corvina (*Cynoscion xanthulus*), gulf croaker (*Bairdiella icistius*), and sargo (*Anisotremus davidsoni*). The fourth species, the tilapia hybrid, is a freshwater-reared euryhaline species that is farmed in nearby aquaculture operations. Costa-Pierce and Doyle (1997) reported that the tilapia population that resides in the Salton Sea was genetically similar to tilapia raised for aquaculture on the eastern shore of the Sea. The tilapia invasion is thought to have occurred following heavy rains in the mid-1960s, however, regular occurrence in the nets of fisherman was not observed until the mid-1970s (Riedel et al., 2002). By the late 1970s this species had become the dominant fish in the Sea.

Hanson (1970) was the first to assemble salinity tolerance data for species currently found in the Salton Sea (Table 1.1). While these values represented upper limits of salinity tolerance based on mortality, it was determined that the optimal salinity with respect to growth, food assimilation and respiration for these species ranged between 33-37 g/l (Brocksen and Cole, 1972). Orange-mouth corvina, sargo, and gulf
croaker are marine fish, and likely do not encounter large changes in the salinity of their native environment; Brocksen & Cole (1972) hypothesized that these species may experience problems maintaining populations if salinity of the Salton Sea reached 40g/l.

Table 1.1. Salinity tolerances of four fish species inhabiting the Salton Sea, California. Values given are for the upper tolerance limit when salinity is increased in gradual increments (see individual papers for methods), and limits to reproduction, if available.

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<th>Species</th>
<th>Upper Salinity Limit</th>
<th>Reproductive Limit</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange-mouth corvina</td>
<td>57.5-62.5 g/l</td>
<td>N/A</td>
<td>Hanson 1970</td>
</tr>
<tr>
<td>(Cynoscion xanthulus)</td>
<td></td>
<td></td>
<td>Brocksen and Cole 1972</td>
</tr>
<tr>
<td>Croaker</td>
<td>58.0 g/l</td>
<td>N/A</td>
<td>Hanson 1970</td>
</tr>
<tr>
<td>(Bairdiella icistius)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sargo</td>
<td>55-57.5 g/l</td>
<td>N/A</td>
<td>Hanson 1970</td>
</tr>
<tr>
<td>(Anisotremus davidsoni)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mozambique tilapia</td>
<td>120 g/l</td>
<td>49 g/l</td>
<td>Stickney 1986</td>
</tr>
<tr>
<td>(Oreochromis mossambicus)</td>
<td></td>
<td></td>
<td>Reidel et al 2000</td>
</tr>
</tbody>
</table>

While the Gulf of California species were introduced at a time when salinity of the Salton Sea was similar to that of the Pacific Ocean (33-35 g/l), the tilapia hybrid invaded by a separate mechanism over a decade later (see below).

Beginning in 1992, the Salton Sea fishery was plagued by large-scale mortality events that over the following years would become larger and more alarming (Hurlbert et al., in press). By 2002 some estimates of fish loss in the Salton Sea were as high as 90%, and there were peaks in mortality occurring semi-annually during both summer and winter months. While salinity tolerance limits of the four species in the fishery were still above the salinity of the Salton Sea proper, the additive effects of several other stressors prevalent in the Sea may have reduced salinity tolerance. In the Salton Sea, there are dramatic seasonal changes in dissolved oxygen (0-20 mg/l), temperature (13 to 35°C), ammonia (NH₃; as high as 8.0 mg/l), and hydrogen sulfide (HS⁻; as high as 5 mg/l).
Mechanisms of Fish Osmoregulation

Teleost fish maintain the total osmolality of the plasma at approximately one-third that of seawater, and therefore face an environmental osmotic gradient when inhabiting freshwater (FW) or seawater (SW). FW-acclimated fishes must compensate for constant osmotic water gains and diffusive ion loss, as a result drinking rate is negligible and they produce a large volume of dilute urine. To offset ion losses, specialized cells on the branchial epithelium called mitochondrial-rich cells (MRC; also referred to as chloride cells) expend metabolic energy in the form of ATP to excrete $\text{Na}^+$ and $\text{Cl}^-$ across the gills against their respective gradients (Keys and Wilmer, 1932; Perry, 1997; Wilson and Laurent, 2002). In contrast, SW fishes drink to offset osmotic water loss, and $\text{Na}^+$ and $\text{Cl}^-$ are actively excreted by a distinctly different MRC sub-type, again at the expense of metabolic energy (Marshall, 2002). MRCs are generally located on the afferent edge of gill filaments with a special concentration in the interlamellar regions of filament epithelium. These cells have been characterized by abundant mitochondria, associated with an extensive network of tubules representing a continuation of the basolateral cell
membrane that possesses high concentrations of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase (Laurent and Dunel, 1980). The mechanism of ion excretion via MRCs is still not fully understood. The most recent model was reviewed by Marshall (2002), with ion excretion driven by a basolateral membrane bound Na\textsuperscript{+}, K\textsuperscript{+}-ATPase (NKA). Because K\textsuperscript{+} tends to rapidly diffuse back out of the cell, NKA essentially functions as a sodium pump, and in most teleost species, along with the apical secretion of Cl\textsuperscript{-}, generates a trans-epithelial potential great enough to drive sodium across the epithelium through paracellular junctions between MRCs and their accessory cells (Marshall, 2002). Chloride excretion is also sodium driven by the Na\textsuperscript{+}, K\textsuperscript{+}, 2Cl\textsuperscript{-} co-transporter (NKCC). Once chloride is in the MRC, it diffuses down a concentration gradient to the outside environment via the cystic-fibrosis transmembrane conductance regulatory protein (CFTR) (Marshall, 2002).

Based on the expression and/or location of NKA, NKCC, and CFTR in tilapia exposed to FW and SW, Hiroi et al., (2005) divided MRCs into four classifications. Type I cells were small and contain only NKA on the basolateral membrane, this cell type was speculated to be what has previously been described as an immature cell. Type II cells expressed NKA on the basolateral membrane, but also an apical NKCC. These were speculated to be involved in FW tolerance, but were an unexpected finding as NKCC had previously been speculated to be found solely in SW. Type III cells expressed basolateral NKA and NKCC, while type IV were identical but also expressing apical CFTR, which supports the current model for SW ion excretion (Hiroi et al., 2005).
Four stages of the MRC life cycle have been identified (van der Heijden et al., 1999; van der Heijden et al., 1997a; Wendelaar Bonga and van der Meij, 1989) and include 1) large columnar mature cells, which had an ultrastructure that is typical of an actively functioning cell, 2) slim crescent-shaped accessory cells, which were less abundant in mitochondria and have a poorly developed tubular reticulum, 3) tear-shaped immature cells, a structural intermediate between mature and accessory cells, and 4) degenerating or apoptotic cells, which had a highly condensed cytoplasm, multi-lobular, heterochromatic nucleus, and a dilated tubular reticulum and mitochondria. In SW-acclimated fishes, mature cells were found in multi-cellular complexes with accessory cells, as well as other mature cells grouped together and forming an apical crypt (van der Heijden et al., 1999; van der Heijden et al., 1997; Wendelaar Bonga & van der Meij, 1989).

Two sub-types of MRCs were observed along the filament (Pisam et al. 1987), which later were identified as having different functional roles (Pisam et al. 1993, Pisam et al. 1995). These two distinct cell types are distinguishable by the binding of fluorescently-labeled peanut lectin agglutin (PNA) (Goss et al. 2001). PNA+ MRCs, similar to the beta cells of the mammalian kidney, are responsible for base regulation and chloride uptake in FW fishes, while PNA- cells regulate acid excretion and sodium uptake and are analogous to the alpha cells of the mammalian kidney (Galvez et al. 2002, Reid et al., 2003). Upon exposure to seawater, PNA+ MRCs from euryhaline fishes have been shown to undergo a morphometric change and remain in the epithelium in an ion excretory capacity, while the PNA- cells disappear (Pisam et al. 1987), and newly differentiated MRCs are also integrated. Hwang (1987) noted that MRCs from seawater-
acclimated fish possessed cytoplasmic digitations at their apical regions that formed shallow junctional links to the external medium. These junctions are thought to facilitate the paracellular pathway by which $\text{Na}^+$ is extruded and usually develop between 12-24 hours following exposure to 20 g/l in tilapia (Hwang, 1987; Marshall, 2002). In addition, Hiroi et al. (2005) found that there was a dramatic transition of type III to type IV MRCs in the tilapia epithelium during this same time course. The number of mature MRCs increased upon transfer to a higher salinity (Foskett et al., 1981; Kültz and Onken, 1993; Zadunaiski et al., 1995), with proliferations most likely due to maturation of immature cells (Foskett et al., 1981; Kültz and Onken, 1993). This was also supported by Hiroi et al. (2005), as type I MRCs matured into type IV cells within 72 h of SW transfer. MRCs also hypertrophy upon exposure to higher salinity, which resulted in an amplification of the basolateral membrane that contains the NKA driving salt-secretion mechanisms (Kültz & Jurss, 1993; Kültz & Onken, 1993) and tends to increase in area in proportion to NKA activity (Karnaky et al., 1976).

Using Mozambique tilapia (*Oreochromis mossambicus*), Foskett et al. (1981) showed that MRCs proliferated for the first 3 d following a salinity increase, and then underwent a hypertrophy from 4-7 d. These authors suggested that MRC hypertrophy resulted from increased synthesis and subsequent incorporation of plasma membrane into the basolateral tubular system. Cioni et al. (1991) found MRCs of Mozambique tilapia adapting to seawater developed larger basolateral surface area relative to the Nile tilapia (*Oreochromis niloticus*), which may have further contributed to their higher degree of salinity tolerance. The Nile tilapia has a maximum salinity tolerance of approximately 43 g/l, approximately one-third that of the Mozambique tilapia. Despite the large disparity
in salinity tolerance between these species, the number of MRCs did not significantly
differ when both species were exposed to elevated salinities (Cioni et al., 1991).

MRCs of both FW and SW sub-types can be found in some euryhaline organisms
regardless of ambient salinity, and the appropriate cell types are exposed to the external
medium based on ambient salinity. Van der Heijden et al. (1997) found that the
percentage of MRC apical crypts exposed to the outside medium increased from 55 to
80% upon exposure to SW. The reverse was seen with prolactin treatment, which was
used in order to simulate FW acclimatory conditions (Agustsson et al., 2003; Borski et
al., 2001; Riley et al., 2003). Respiratory pavement cells have been observed covering
the MRC subtype that is not needed for the immediate environment, which has been
referred to as morphological oscillation. Daborn et al. (2001) observed morphological
oscillations in vitro by altering the osmolality of the bathing solution on the serosal
surface of the opercular membrane. Furthermore, Hiroi et al. (2005) found that in
addition to closing of apical crypts, CFTR was removed from the membrane immediately
following SW to FW transfer in Mozambique tilapia. While a great deal is known
regarding osmoregulation in FW, SW, and FW to SW transfer, far less is known about
the mechanisms associated with exposure to salinities greater than SW.

Energetics

It has been widely hypothesized that metabolic rate will increase when the
ambient salinity differs from that of plasma, due to an increased demand for ion
regulation, which has been observed during investigations with several species including
Mozambique tilapia (Febry and Lutz, 1987), Nile tilapia (Farmer and Beamish, 1969),
and rainbow trout (*Oncorhynchus mykiss*; Rao, 1968). In each species, metabolic rate was reduced when the gradient between the plasma and the environment was the lowest, and was increased in FW and SW (Morgan and Iwama, 1991). In one previous study, an elevated growth rate was observed in fishes exposed to isosmotic conditions (Boeuf and Payan, 2001).

Morgan et al. (1997) found that whole-animal oxygen consumption rate (\( \dot{M}O_2 \)) after four days of exposure to 25 g/l salinity were significantly elevated in Mozambique tilapia relative to fish in isosmotic or FW. The peak in oxygen consumption rate coincided with increased \( \text{Na}^+, \text{K}^+\)-ATPase activity and growth hormone levels, and it was hypothesized that 20% of metabolic energy was involved with osmoregulation in tilapia after 4 d of exposure to 25 g/l. However, in studies using the isolated gills of FW and SW-acclimated cutthroat trout (*Oncorhynchus clarki clarki*) the energetic cost of NaCl transport was calculated to be less than 4% of the animals total energy budget (Morgan and Iwama, 1999). Gill tissue oxygen consumption rate was measured by Morgan and Iwama (1999) with and without the NKA-inhibiting compound oubain, so these values represented the energy demand for maintenance of the mechanism of NaCl excretion and not that of the cellular rearrangements necessary when switching between FW and SW acclimation. Morgan and Iwama (1991) summarized three additional patterns of metabolic rate observed in euryhaline or anadromous species during exposure to increased salinity. The first of which is a proportional increase in metabolic rate with salinity; this is seen in juvenile rainbow trout and juvenile Chinook salmon (*Oncorhynchus tshawytscha*). Second, sub-adult Chinook salmon and killifish (*Fundulus heteroclitus*), have a metabolic rate that does not significantly change over a range of
salinities. Lastly, species of the genus *Cyprinodon* have metabolic rates at a minimum in seawater, with an increase at lower salinities (Morgan and Iwama, 1991). Farmer (1969) found no difference in the slopes of lines representing salinity and oxygen consumption in Nile tilapia. Results were similar with rainbow trout (Rao, 1968) and milkfish (*Chanos chanos*), where animals held in 55 g/l seawater consumed oxygen at rates indistinguishable from values measured in fish exposed to isosmotic water. Swanson (1998) suggested that the increased demand was compensated for by a reduction in activity level. In the Eustis pupfish (*Cyprinodon variegatus hubbsi*), ion regulation does not appear to alter energetic costs when measured at the whole organism level (Jordan et al., 1993). In summary, there are conflicting reports of the effect of salinity on metabolic rate in different species that have been measured by different researchers. Investigation into how salinities greater than SW effect the metabolic rate of tilapia had not been measured prior to the research reported in this thesis.

**The Osmo-respiratory Compromise**

The gills of fishes play a central role in gas exchange, ion regulation, acid-base balance and nitrogenous waste excretion. Consequently, there are trade-offs and compromises in gill design and function so that these processes can occur across one structure sufficiently and in some cases optimally. The morphological design of the gill has been previously reviewed (Laurent, 1984), where a clear regional separation of osmoregulatory and respiratory functions was evident. Gas exchange takes place primarily across the lamellar epithelium, while oxygen and carbon dioxide move via simple diffusion across flattened squamousal cells called pavement cells (PVCs). While...
PVCs are found throughout the branchial epithelium, they are the dominant cell type on the surface of the lamellae in the majority of fish species. The filamental epithelium has a more mosaic design, containing MRCs, mucous-secreting cells (goblet cells) and PVCs. Furthermore, it has been noted that MRCs tend to be more localized to the afferent filamental epithelium, while the efferent tends to be more mucous-cell rich.

Oxygen and carbon dioxide are exchanged across the gills via simple diffusion, where exchange rate is a function of the partial pressure gradient for the respective gases, diffusional surface area, diffusion distance, and permeability of the barrier. Gas flux across the gills can be increased, under conditions such as exercise, by elevating water ventilation rate as well as blood flow through the gills (Randall, 1970; Stevens and Randall, 1967). The thin-walled lamellae are held open by muscular pillar cells supporting columns of collagen that can be adjusted in length in response to lamellar blood flow patterns. Increased blood pressure results in further recruitment of lamellae (Farrell et al., 1979), and increases the functional surface area for diffusion by reducing the blood to water diffusion distance. In general, diffusion rate is maximal when diffusion distance is low, surface areas are large, and permeability is high. These characteristics are in direct contrast with those that minimize ion movements across the gills. Alterations in both metabolic rate and osmotic balance can be potentially constrained due to compromises between maintaining sufficient gas transfer and defending water loss.

The compromise between osmoregulation and respiration was noted by Randall et al. (1972), although Nilsson (1986) later referred to it as the osmo-respiratory compromise. To test the hypothesis that enhanced gas transfer would increase ion
Randall et al. (1972) monitored the flux of radio-labeled sodium (Na\textsuperscript{22}) in FW rainbow trout (Oncorhynchus mykiss) that had been exercised, or injected with either noradrenaline or isoprenaline. All three treatments increased sodium efflux, with the bout of exercise resulting in the greatest rate of sodium loss. Catecholamine stimulation resulted in about 50% of the efflux observed during exercise. The hypothesis of Randall et al. (1972) was that changes in sodium loss were catecholamine mediated, and resulted from alterations in blood flow patterns through the gills associated with increased oxygen demand. Subsequent work by Wood and Randall (1973), where multi-directional sodium fluxes were investigated during exercise and recovery in the same species. Activity was imposed upon the fish by chasing, and resulted in increased ventilation and perfusion of the gills. During activity, sodium efflux increased approximately 70%, resulting in a net sodium loss. Once again, changes in gill blood flow were thought to result from lamellar recruitment and subsequent diffusive ion loss in freshwater. Gonzalez and McDonald (1992) investigated the relative change of sodium efflux to $\dot{M}O_2$ (the ion-gas ratio; IGR) in FW-acclimated rainbow trout following osmotic challenge, low ambient [Ca\textsuperscript{2+}], and exercise. As predicted, increases in $\dot{M}O_2$ corresponded with increased ion efflux. The authors concluded that the increased $\dot{M}O_2$ was associated with increased gill functional surface area, decreased diffusion distance resulting from lamellar thinning, and increased O\textsubscript{2} permeability mediated by catecholamines; all of which were expected to result in increased ion losses proportional to the increases in $\dot{M}O_2$. Interestingly, ion flux and $\dot{M}O_2$ did not increase proportionately, resulting in an increase in the IGR from 1.6-10X. The increase in IGR was attributed to distortion of paracellular tight junctions along the respiratory epithelium (Gonzalez and McDonald, 1992) as this increased ion loss without
affecting gas flux. Investigating the effects of exercise training, Gallaugher et al. (2001) swam both trained and untrained SW-acclimated Chinook salmon (*Oncorhynchus tshawytscha*) at $U_{\text{crit}}$ and 80%$U_{\text{crit}}$ and measured the changes in plasma osmolality. They found that trained fish once again appeared to be able to achieve higher levels of $\dot{M}O_2$, but had significantly lower plasma osmolality at both swimming speeds relative to their untrained counterparts. The beneficial effect of exercise training on osmoregulation in SW fish may have its basis at the level of the gut rather than the gill. In a SW-acclimated fish, the gut plays a large osmoregulatory role, as it actively absorbs ions from imbibed water, to drive water uptake. Trained fish show less of a reduction in gut blood flow during exercise than untrained fish, and thus trained fish may be more capable of water absorption across the gut to combat loss at the gill (Gallaugher et al. 2001). The osmorespiratory compromise clearly places limitations on both functions. In the Salton Sea, variables such as low oxygen availability and/or high temperature have direct effects on metabolism, resulting in alterations in perfusion and ventilation of the gill. Mediated by the osmorespiratory compromise, these conditions may place severe restrictions on the ability to effectively osmoregulate in a very saline environment.

**Temperature/Salinity Interactions**

Large seasonal and daily fluctuations in temperature have been observed in the Salton Sea (Watts et al., 2001). Temperature increases and decreases can have deleterious effects on osmoregulation by altering enzyme kinetics (Handeland et al., 2000), varying phospholipid membrane composition (Johnston and Cheverie, 1985), and by altering metabolic rate, which can intensify the effects of the osmo-respiratory
compromise (Barron et al., 1987; Hayton and Barron, 1990). Gonzalez and McDonald (1994) suggested that the need to defend ion balance is what sets limits to metabolic scope, as increased metabolic rate leads to increased gill perfusion and functional surface area, but enhances the potential for osmoregulatory disturbances.

Studies investigating the interaction of temperature and salinity are few, and even fewer are those investigating how salinities greater than SW confound this interaction. Staurnes et al. (2001) found that Atlantic salmon smolts (Salmo salar) acclimated to 8°C had an increased plasma chloride level when transferred to SW at 5 or 14°C, which increased even further when exposed to 2 and 18°C. In three-spined sticklebacks (Gasterosteus aculeatus) acclimated to FW, Schaarschmidt et al. (1999) found a lower level of tissue taurine in fish acclimated to 4 vs. 15°C. Taurine is an intracellular organic osmolyte found in this species, and heavy mortality in sticklebacks exposed to 4°C was attributed to osmoregulatory failure (Schaarschmidt et al., 1999). Robertson & Hazel (1999) found similar results in rainbow trout (Oncorhynchus mykiss) as in tilapia isolated gills, with water weight gain reduced following acclimation to elevated temperature (5°C versus 20°C in RBT). Furthermore, 5°C-acclimated rainbow trout had a higher level of membrane cholesterol, which decreases the fluidity of the phospholipid bilayer and restricts the formation of voids within the membrane that are commonly observed during elevated temperature stress; these voids have been implicated in osmoregulatory failures (Robertson & Hazel, 1999).

As a tropical fish, one disadvantage California Mozambique tilapia may have relative to other Salton Sea fishes is the lack of ability to tolerate low temperatures (Al Amoudi et al., 1996). The fish species transferred from the Gulf of California (corvina,
croaker, sargo) have most likely historically experienced similar low temperatures as those recorded in the Salton Sea (e.g. 13°C), but California Mozambique tilapia, although relatively eurythermal, experience low temperature induced osmoregulatory failure in freshwater (Al Amoudi et al. 1996). Based on the high salinity of the Salton Sea, in addition to osmoregulatory challenges associated with winter temperatures observed in the Salton Sea (as low as 13°C), the interaction between temperature and salinity may be a potential cause for tilapia die-offs that are observed during the winter months at the Salton Sea.

Outline of Thesis

In order to test the idea that multiple stressors such as salinity and temperature are responsible for the wide-scale mortality of the California Mozambique tilapia at the Salton Sea, I will address two objectives:

I) To characterize the response of tilapia hybrids to salinities above seawater (Chapter 2), and investigate the mechanisms behind previously documented tolerance for extreme salinities in this species (Chapter 3) in order to understand how this physiological system functions under osmotic stress.

II) To investigate the effects of temperature on the osmoregulatory ability of this tilapia species during exposure to a range of salinities (Chapters 4, 5, and 6).

Objective 1: In chapter 2, tilapia were gradually exposed to progressively increased salinities, ranging from 35 to 95 g/l and sampled at 0, 1 and 5 days. Although the ionic
composition differs with respect to the level of calcium and sulphate, artificial sea salts were used to create hypersaline challenges (see Appendix I). Physiological (plasma osmolality, $[\text{Na}^+]$, and $[\text{Cl}^-]$), muscle water content, drinking rate, and oxygen consumption rate), biochemical (NKA activity), and morphological (MRC number and stage) indicators of osmoregulatory stress were quantified. These data provided an in depth characterization of how tilapia respond to salinity increases, and provided a framework for modeling of salinity tolerance of this species with respect to the progressively increasing salinity of the Salton Sea.

In Chapter 3, tilapia were exposed to a wider range of salinities relative to chapter 3 (FW-75 g/l), and were held at target salinities for up to 28 days (sampled at 1, 5, 14, and 28 d). I measured the physiological indicators of osmoregulatory stress as described above, as well as the activities of NKA and/or total ATPase in muscle, liver, intestine, kidney, brain, and gill. These latter variables were correlated with whole animal $\dot{M}O_2$ in order to investigate the possibility of a biochemical basis for salinity-induced metabolic reductions.

**Objective 2:** Chapter 4 is an assessment of how changes in ambient temperature affect the responses of tilapia to salinity characterized in chapters 2 and 3. I used 24 h temperature-salinity challenges, where fish acclimated to 35 g/l at 25°C were directly transferred to 35, 43, 51, or 60 g/l at 15, 25, or 35°C. Plasma osmolality and muscle water content were used as indicators of hypoosmoregulatory ability. The interaction between temperature and salinity was further investigated in chapter 5, where responses of tilapia acclimated to the salinity of the Salton Sea at 25 °C were measured following direct transfer to 15 or 35°C at constant salinity. A time course was also developed for
the 24 h challenge of a transfer from 35 g/l at 25°C to 43 g/l at 15 or 35°C. Plasma osmolality, muscle water and NKA activity were measured following this challenge. Conditions used in this experiment were used to draw conclusions regarding how tilapia may respond to these conditions in the Salton Sea, with notes on the potential role of temperature and salinity interactions in the fish kills that plague the Salton Sea fishery. Chapter 6 is a thorough description of how temperature acclimation affects MRC characteristics. Tissue microarrays and laser scanning cytometry were used to describe changes in cell size, cell number, and the abundance of NKA in both FW and SW exposed tilapia at 15, 25, and 35°C. In addition, the effects of acclimation temperature on the kinetics of branchial and intestinal NKA activity were investigated using multiple assay temperatures.
References


PART ONE: RESPONSES OF TILAPIA TO HYPERSALINE EXPOSURE
CHAPTER 2: PHYSIOLOGICAL, BIOCHEMICAL, AND MORPHOLOGICAL INDICATORS OF OSMOREGULATORY STRESS IN CALIFORNIA MOZAMBIQUE TILAPIA (*Oreochromis mossambicus* x *O. urolepis hornorum*) EXPOSED TO HYPERSALINE WATER.

Introduction

Saline lakes exist on every continent worldwide, and comprise a total volume of approximately 104,000 km$^3$ (Williams, 1996). The Salton Sea is a 980 km$^2$, highly saline lake that formed in 1905-06 when Colorado River water flooded the Imperial Valley of south-eastern California. This inland sea has a high evaporation rate and lacks outflow; consequently, salinity has continually risen since its formation. The current salinity is approximately 43 g/l, but is increasing by 0.3 g/l/year (Watts et al., 2001). Because reproductive failures and high larval mortality among introduced marine fish species have been partially attributed to high salinity (Costa-Pierce and Riedel, 2000), the continual salinity increase has generated concern due to the threat it places on the current fishery. In spite of this challenge, one species that has successfully inhabited the sea is a Mozambique-Wami tilapia hybrid (*Oreochromis mossambicus* x *O. urolepis hornorum*), which has been referred to as the ‘California’ Mozambique tilapia (Costa-Pierce and Doyle, 1997). Introduction of this tilapia was reported to have occurred in 1964-65, when hybrids from local aquacultural farms escaped to drainage ditches leading into the sea (Costa-Pierce and Doyle, 1997). Although Mozambique tilapia are typically freshwater and estuarine, they have been observed to tolerate very saline water during experimental short term direct transfers (Kültz et al., 1992; Kültz and Onken, 1993; Stickney, 1986);

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very little is known regarding the salinity tolerance of the Wami tilapia (*Oreochromis urolepis hornorum*), but they have been reported to be able to grow and reproduce at salinities ranging up to 39 g/l (Talbot and Newell, 1957). This study will be the first to address the saline tolerance of the California hybrid, which is a hypersaline lake inhabitant.

Teleosts exposed to hyperosmotic environments experience osmotic water loss coupled with diffusive ion gains. To offset the loss of water, an increase in drinking rate becomes necessary (Brocksen and Cole, 1972; Foskett et al., 1981; Hwang et al., 1989; Cioni et al. 1991); in the intestine water is absorbed osmotically, following the actively absorbed salts across the epithelium (Wilson et al. 1996). In order to offset the increased ion load, and ultimately survive in saline waters, fish must actively excrete Na\(^+\) and Cl\(^-\). Gills are the primary organ involved in osmoregulation in teleost fishes. Ion-transporting mitochondrial-rich cells (MRCs) are located on the branchial filamental epithelium, and are rich in Na\(^+\), K\(^+\)-ATPase, which drives excretion mechanisms. MRCs are responsible for ion excretion in both juvenile and adult seawater-acclimated fish (Foskett et al., 1981; Laurent and Dunel, 1980; Marshall and Bryson, 1998; Perry, 1997). MRCs are generally rich in mitochondria, have an extensive basolateral tubular reticulum, and have direct contact with ambient water through the apical membrane and with blood through the basolateral cellular membranes (Laurent, 1984; van der Heijden et al., 1997; Wendelaar Bonga and van der Meij, 1989). Four stages of the MRC life cycle have been identified, and include 1) large columnar mature cells, which have an ultrastructure that is typical of actively functioning cells, 2) slim crescent-shaped accessory cells, which are less abundant in mitochondria and have a poorly developed tubular reticulum, 3) tear-shaped
immature cells, structurally intermediate between mature and accessory cells, and 4) small and round degenerating, or apoptotic cells, which have a highly condensed cytoplasm, multi-lobular and heterochromatic nucleus, and dilated mitochondria and tubular reticulum (Wendelaar Bonga and van der Meij, 1989; Wendelaar Bonga et al., 1990). Salinity tolerance in fishes is dependent upon the appropriate physiological, biochemical, and morphological adjustments to a given salinity.

The salinity tolerance of pure Mozambique tilapia (*Oreochromis mossambicus*) has been previously investigated, with the primary focus being on seawater acclimation (35 g/l), or hypersaline (>35 g/l) acclimation following large increases in salinity (i.e. direct transfers). This study investigated the ability of a hybrid of this euryhaline species to gradually acclimate to salinities exceeding that of seawater (up to 95 g/l), and incorporates physiological (plasma osmolality, [Na⁺], and [Cl⁻], oxygen consumption, drinking rate, hematocrit, mean cell hemoglobin concentration, and muscle water content), biochemical (Na⁺, K⁺-ATPase), and morphological (the number of mature, accessory, immature and apoptotic MRCs) indicators of osmoregulatory stress. The ultimate goal of this study was to identify the most appropriate variable that can be used to model salinity tolerance for fish species that inhabit hypersaline lakes, using a species that is currently known to inhabit such an environment.
Materials and Methods

Fish

Pacific Aquafarms in Niland, California donated 300 juvenile tilapia hybrids (Oreochromis mossambicus x O. urolepis hornorum), which were transported to San Diego State University where all experiments were conducted. Tilapia averaging 36.1 ± 0.4 g, were fed commercial trout food daily, except within 24 h prior to sampling.

Approximately 30 seawater-acclimated fish were held in each of ten 60 l glass aquaria maintained at 23-25°C; aquaria were fitted with charcoal filters and air stones. Tilapia were acclimated stepwise to 35 g/l over three transfers (0-10 g/l, 10-20 g/l, and 20-35 g/l), with 4 d allowed for acclimation at each stage, and then allowed to acclimate for two weeks to full strength seawater (35 g/l). Ammo-lock was added to detoxify ammonia, and three-quarters of the water volume was changed every 2.5 d. Seawater was prepared using Instant Ocean synthetic sea salt in dechlorinated tap water, and salinity was measured using a light refractometer.

Experiment I: 28 day acclimation to seawater (35 g/l)

To determine the time course for seawater-acclimation in this hybrid, two tanks of fish were kept at 35 g/l salinity for 28 days, and seven fish were removed and sampled at 0, 24 (1 d), 120 (5 d), 336 (14 d), 504 (21 d), and 660 h (28 d) following transfer for measurement of plasma osmolality, [Na⁺], and [Cl⁻], muscle water content, Hematocrit (Hct) and mean cell hemoglobin concentration (MCHC). Fish sampled at 14 d were also sampled for Na⁺, K⁺-ATPase activity, and gill morphological parameters.

Tilapia were anaesthetized using 0.7 g/l benzocaine, which was first dissolved in 3 ml of ethanol. Fish were patted dry and weighed, and the caudal peduncle was severed.
Blood was collected into heparinized microhematocrit capillary tubes for measurement of Hct and total hemoglobin. Approximately 1.5 g of the left dorsal epaxial muscle was removed to determine muscle water content. Muscle tissue was placed into pre-weighed, plastic scintillation vials and weighed prior to, and following drying to constant weight for 72-96 hours at 70°C.

The second and third right gill arches and approximately 1.5 cm of duodenal intestine were removed, frozen on dry ice and stored at -80°C for later analysis of Na⁺, K⁺-ATPase activity. The second and third left gill arches were removed from the right side of five fish at the 120 h sampling time, and immediately fixed in cold Karnofsky's fixative for at least two hours. The middle third of each gill arch was cut off by a razor blade, and gill arches about 1 mm long, bearing up to 20 filaments in both anterior and posterior rows were used for SEM, while individual filaments were cut for TEM and LM-studies. All specimens were rinsed three times for 10 min each in the phosphate-buffered saline (PBS), and post-fixed in 1% osmium tetroxide for 1 h.

Experiment II: Direct transfer from seawater to 60 and 85 g/l

Thirty seawater-acclimated tilapia were directly transferred to salinities of 60 and 85 g/l, via a three-quarter tank water change. Seven fish from each tank were sampled at 0, 3, 24, and 120 h following transfer. Fish from all sampling times were sampled for measurement of plasma osmolality, [Na⁺], and [Cl⁻], muscle water content, Hct and MCHC, while those samples at 120 h were also sampled for Na⁺, K⁺-ATPase activity; sampling was conducted as described in Series I.
Experiment III: Gradual salinity increase from seawater to 95 g/l

Seawater-acclimated tilapia in the remaining tanks were gradually exposed to increased salinity. Salinity in all tanks was increased 10 g/l every five days, via a three-quarter water change, up to 95 g/l; this yielded experimental salinities of 45, 55, 65, 75, 85, and 95 g/l. At each salinity, seven fish were sampled at 0, 3, 24, and 120 h. Fish from all sampling times were sampled for measurement of plasma osmolality, [Na$^{+}$], and [Cl$^{-}$], muscle water content, Hct and MCHC, while those samples at 120 h were also sampled for Na$^{+}$, K$^{+}$-ATPase activity and morphology; sampling was conducted as described Series I.

Experiment IV: Oxygen Consumption and Drinking Rate

Oxygen consumption rate ($\dot{M}O_2$) and drinking rate were measured in fish acclimated to 35, 55, 75, and 95 g/l. $\dot{M}O_2$ was measured according to the methods of Gonzalez and McDonald (1994). Following measurements at 35 g/l, salinity was increased 10 g/l every four days until the next target salinity was reached. Fish were left at the target salinity for one week before measurements were made, after which salinity was increased again as previously described. When measuring $\dot{M}O_2$, six fish were weighed and transferred to individual 140 ml chambers that were connected to a 60 l recirculating system filled with water of the appropriate salinity. Flow rate into individual chambers was approximately 100 ml/min, and fish were allowed to recover for 24 h before measurements were taken. To initiate measurements, water samples were withdrawn from each chamber while water was still flowing. Flow was then immediately stopped and the chambers were sealed. After 15 min, a second water sample was taken.
and flow was restored. The initial and final water samples were analyzed for $PO_2$ with a Cameron $O_2$ meter. Using the initial and final $PO_2$, and chamber volume, as well as fish mass, time, and the oxygen absorption coefficient for the target salinity, moles of $O_2$ consumed per unit time were calculated. Two sets of six fish were measured on consecutive days.

Drinking rate was measured using a modified method of Wilson et al. (1996). Fish were placed in individual 250 ml chambers connected to a re-circulating system filled with 50 L of the appropriate salinity at least 24 h prior to the beginning of the measurement period. Five microcuries (185 kBq) of tritiated PEG was added to each chamber and 5 ml water samples were taken after 4 and 8 h. After 8 h fish were killed using MS-222, rinsed, weighed and ligated gut was removed. The whole guts were put in 4 ml 8% perchloric acid, homogenized for one minute, and centrifuged. The supernatant and water samples were assayed for radioactivity. Drinking rate was calculated from the average activity per ml of water over the 8 h and the total number of counts taken up by the gut in that period.

Analyses
--Blood Variables

Heparinized microhematocrit capillary tubes were centrifuged at 11,500 RPM for 3 min with a Damon IEC MB microhematocrit centrifuge. Hematocrit (Hct) was recorded in duplicate or triplicate depending on available blood volume and plasma was expelled into Eppendorf tubes and frozen at -80°C for analyses of plasma osmolality, $[Na^+]$ and $[Cl^-]$. Whole blood total hemoglobin concentration ([Hb]) was measured using
a Sigma total hemoglobin assay kit, with absorbance measured using a Beckman DU 640 spectrophotometer at 540nm; mean cell hemoglobin concentration (MCHC) was calculated as [Hb]/(Hct/100). Plasma osmolality was measured using a Wescor 5500 vapor pressure osmometer, and expressed as milliosmoles per kilogram water (mOsm/kg H₂O). Plasma [Cl⁻] was measured using the calorimetric mercuric thiocyanate method (Zall et al. 1956), and plasma [Na⁺] was measured with an atomic absorption spectrophotometer (Perkin Elmer model 3100 A). Plasma [Na⁺] and [Cl⁻] are expressed as millimoles per liter plasma (mM).

--Na⁺, K⁺-ATPase activity

Gill tissue taken from fish exposed to salinity for 120 h were homogenized in 1 ml of SEJD buffer (250 mM sucrose, 10 mM EDTA-Na₂, 50 mM imidazole, pH 7.3). Na⁺, K⁺-ATPase activity at 120 h was determined using the method of Gibbs and Somero (1990) and expressed as μMol of ADP per hour per mg total protein (μmol/h/mg); protein was determined by the Biuret method (Gibbs and Somero, 1990).

--Scanning electron microscopy.

Fixed specimens were dehydrated in a graded ethanol series, concluding at 100%, critical-point- dried with liquid CO₂, mounted on stubs, sputter-coated with gold-palladium, and examined with a Hitachi S 2700 scanning electron microscope at the accelerating voltage of 10 kV. Areas on the trailing (afferent) surface of filament behind the secondary lamellae and without interlamellar regions were randomly chosen. Photographs at 2000 X magnification of this area (2400 μm²) from ten different filaments
per fish and three fish per group were used for quantification of apical pits and general examination of the superficial structure of filament epithelium.

—Light microscopy and transmission electron microscopy.

Fixed specimens were dehydrated in graded ethanol/acetone series with the final change in absolute acetone before infiltration and embedding in Epon epoxy resin. Longitudinal semi-thin (1 μm) and ultra-thin (60-70 nm) sections, made parallel to the long axis of filaments, were cut using a LKB-microtome. Semi-thin sections were mounted on glass slides, stained with 0.5% methylene blue, and examined on a Diastar microscope. Ultra-thin sections were mounted on the copper grids, double stained with 1% uranyl acetate, followed by 0.5 % lead citrate, and examined in a Philips 410 electron microscope at 80 kV. The ultrastructure of chloride cells was studied in the interlamellar areas of the filament epithelium. The percentage of mature, accessory, immature, apoptotic, and necrotic MRCs was determined, following the method described by (Wendelaar Bonga and van der Meij (1989), on 20 randomly-selected interlamellar areas, in three filaments per fish, and for four fish per group.

Statistics

The effects of salinity on Hct, MCHC, plasma osmolality, [Na⁺], [Cl⁻], muscle water content, and morphological data were analyzed using one-way ANOVA followed by a post-hoc Dunnet’s test. \( \dot{M}O_2 \) and drinking rate were analyzed by one-way ANOVA followed by a post-hoc Tukey test. ANOVAs were performed using JMP (SAS Institute 2000). Alpha values for significance were 0.05 in all statistical tests.
Results

Experiment I: 28 day acclimation to seawater (35 g/l)

--Plasma Osmolality, [Na\(^+\)], and [Cl\(^-\)]

No mortality was observed in tilapia hybrids during acclimation to seawater. Plasma osmolality, [Na\(^+\)] and [Cl\(^-\)] were significantly elevated 24 h following transfer from 20 to 35 g/l salinity; however, after 24 h plasma osmolality decreased to a consistent level, not significantly different from pre-transfer values (Fig. 2.1). Plasma [Na\(^+\)] and [Cl\(^-\)] remained at elevated, but consistent levels throughout the remainder of the experiment. There were no changes in muscle water content at any time throughout the 28 d exposure (pooled mean ±SE = 80.6±0.1%), and hematological parameters such as Hct and MCHC remained unaffected as well (pooled means ±SE = 30.4±0.3% and 3.4±0.03 [Hb]/Hct/100, respectively).

--Morphology

Following 2 weeks of exposure to 35 g/l, pavement cells, a predominant component of the outer layer of filamental epithelium, displayed both long and short microridges organized into a concentric pattern with a flat central area. Numerous deep apical pits of multicellular MRC complexes and few pores of mucous cells were observed on the surface of filamental epithelium (Fig. 2.2B). MRC complexes combined mature, immature and accessory cells that were linked by short apical tight junctions and followed desmosomes (Fig. 2.3A-B). No interdigitations between apexes of MRCs within the multicellular complexes were found. Aging MRCs degrading by apoptosis displayed a reduced size, highly condensed cytoplasm, a markedly indented nucleus with
condensed chromatin, and numerous enlarged mitochondria surrounded by distended
tubules of the network (Fig. 2.3D). Numbers of mature, accessory, immature, and
apoptotic cells are presented in Table 2.1.

Experiment II: Direct transfer from seawater to 60 and 85 g/l

Direct transfer from 35 to 85 g/l salinity resulted in 100% mortality. In contrast,
all fish survived direct transfer to from 35 to 60 g/l, but significant effects on sub-lethal
indicators of osmoregulatory stress were observed. Plasma osmolality, [Na\(^+\)], and [Cl\(^-\)]
increased only qualitatively 3 h post-transfer, while significant increases over pre-transfer
values were observed 24 h after and were only observed in osmolality (Fig. 2.4). Plasma
osmolality, [Na\(^+\)], and [Cl\(^-\)] all returned to near pre-transfer values by 120 h. Na\(^+\), K\(^+\)-
ATPase activity significantly increased from 2.46 ± 0.48 to 8.87 ± 1.62 μmol/h/μg
protein between 0 and 120 h, respectively

Experiment III: Gradual salinity increase from seawater to 95 g/l

--Plasma Osmolality, [Na\(^+\)] and [Cl\(^-\)]

With the exception of minor mortality observed at 85 g/l salinity, tilapia hybrids
survived a 10 g/l salinity increase every 5 d up to a salinity of 95 g/l. Plasma osmolality
values were not significantly different, relative to 45 g/l, in fish exposed to 55-65 g/l
salinity, and were consistent with values obtained from 35 g/l fish sampled at 14 and 28
d. However, in 75 g/l-exposed fish, osmolality was significantly increased at 24 and 120
h following transfer. At the 3h sampling time, plasma osmolality was significantly
increased relative to 45 g/l in fish exposed to salinities of 85 and 95 g/l (Fig. 2.5). Plasma
[Na$^+$] and [Cl$^-$] followed similar trends, and were significantly elevated at 85 and 95 g/l, with the exception of [Cl$^-$] at 3 h, which was only significantly increased at 95 g/l (Fig 2.6).

--Na$^+$, K$^+$-ATPase activity

Branchial Na$^+$, K$^+$-ATPase activity remained relatively constant in gills exposed to 35-65 g/l salinity, but was significantly increased at 75 and 85 g/l (Fig. 2.7). Intestinal Na$^+$, K$^+$-ATPase activity averaged 5.30 ± 0.41 over the range of salinities, and did not vary significantly (data not presented).

-- Morphology

The superficial pattern of pavement cells was largely unchanged from that of a typical seawater-acclimated epithelium in fish exposed to 45-65 g/l salinity (Figs. 2.2 and 2.8A), although the first appearance of interdigitated junctions between mature and accessory MRCs was in fish exposed to 55 g/l salinity (Fig. 2.9). However, at 75-95 g/l salinity the surface of pavement cells bore fewer marginal microridges, and cells had an expanded flat central area (Fig. 2.8B-D). The number of apical pits per 2400μm$^2$ remained relatively consistent in the epithelia of tilapia exposed to 35-55 g/l, but then was significantly increased from 65-95 g/l salinity (Table 2.1). No mucous cells were observed in the filamental epithelium of fish exposed to 75 g/l salinity or greater. The first appearance of interdigitated mature and accessory MRCs was noted in fish exposed to 55 g/l salinity (Fig. 2.9A).
Figure 2.1. The effect of transfer from 25 to 35 g/l seawater on plasma osmolality, [Na\(^+\)], and [Cl\(^-\)] in tilapia hybrids from 0 to 28 days following transfer. * Indicates significant difference relative to 0 h (p<0.001, n=7).

Figure 2.2. SEM showing the surface of gill filaments of tilapia adapted to 35-55 g/l salinity for 14 d. A- Middle of gill filament from 35 g/l-exposed fish; rectangle marks the area examined on the trailing edge (L-secondary lamellae, LE-leading edge, TE-trailing edge). B- Filamental epithelial surface from 35 g/l-exposed fish; arrows indicate of the chloride cell apical pits (MC-mucus cells, PC- pavement cell). C- Filamental epithelial surface in 45 g/l-exposed fish. D- Filamental epithelial surface in 55 g/l-exposed fish; (scale bars: A-100μm, B-D-10μm).
Figure 2.3. TEM showing the mitochondrial-rich cell ultrastructure from the filamental epithelium of tilapia exposed to 35 g/l salinity for 14 d.

A. Interlamellar epithelium (AC-accessory cell, CC-mature MRC, IC-immature chloride cell).
B. Multicellular complex formed by mature and accessory MRCs; arrowhead indicates short apical tight junctions between MC and AC. Arrows indicate long tight junctions between MC and PC (A-apical pit, D-desmosome).
C. Perinuclear cytoplasm of a mature chloride cell (M-mitochondria, TR-tubular reticulum).
D. Apoptotic cell with nuclear and cytoplasm condensation and distension of the tubular reticulum (Scale bars: 1μm).
Figure 2.4. The effect of transfer from 35 to 65 g/l salinity on plasma osmolality, [Na$^+$], and [Cl$^-$] in tilapia hybrids from 0 to 120 h following transfer. * Indicates significant difference relative to 0 h (p=0.01, n=7).

Figure 2.5. The effect of salinity on plasma osmolality in tilapia hybrids following graded transfers to 35, 45, 55, 65, 75, 85, or 95 g/l salinity. Different lines represent sampling times of 3, 24, or 120 h following transfer (see key). * Indicates significant difference relative to 45 g/l (p<0.001, n=7).
Figure 2.6. The effect of salinity on a) Plasma [Cl⁻] and b) Plasma [Na⁺] in tilapia hybrids following graded transfers to 35, 45, 55, 65, 75, 85, or 95 g/l salinity. Different lines represent sampling times of 3, 24, or 120 h following transfer (see key). * Indicates significant difference relative to 45 g/l (p<0.001, n=7).
Figure 2.7. Branchial Na\(^+\), K\(^+\)-ATPase activity measured in tilapia hybrids acclimated to 35, 45, 55, 65, or 85 g/l salinity for 120 h. * Indicates significant difference relative to 35 g/l (p<0.001, n=7).
Figure 2.8. SEM showing the surface of filamental epithelium in tilapia exposed to A) 65 g/l, B) 75 g/l, C) 85 g/l, and D) 95 g/l salinity for 120 h (Scale bars: 10 μm).

Figure 2.9. TEM showing mitochondrial-rich cell ultrastructure in the filament epithelium of tilapia exposed to 45 and 55 g/l salinity for 120 h.
A- Apical pit (A), formed by interdigitated apex of mature MRC and cytoplasmic projections of accessory cells, in fish exposed to 55 g/l salinity; asterisks- cytoplasmic projections of accessory cells, arrowheads- short junctions between MRC and ACs.
B- Mature cell with a deeply indented nucleus (Scale bars: 1 μm).
Table 2.1: Cellular parameters measured under scanning and transmission electron microscopy. Values are presented as mean number of cells ± SE (* significantly different from SW value, p<0.001).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>35 g/l</th>
<th>45 g/l</th>
<th>55 g/l</th>
<th>65 g/l</th>
<th>75 g/l</th>
<th>85 g/l</th>
<th>95 g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of apical pits per selected area (1)</td>
<td>8.4 ± 1.9</td>
<td>9.2 ± 2.7</td>
<td>8.8 ± 2.1</td>
<td>17.0 ± 3.4*</td>
<td>16.9 ± 4.6*</td>
<td>13.6 ± 2.4*</td>
<td>13.1 ± 3.5*</td>
</tr>
<tr>
<td>Number of mature MRCs per selected area (2)</td>
<td>36.7 ± 4.1</td>
<td>35.1 ± 4.0</td>
<td>36.2 ± 3.3</td>
<td>37.2 ± 3.5</td>
<td>20.5 ± 4.5*</td>
<td>14.3 ± 2.6*</td>
<td>11.0 ± 2.9*</td>
</tr>
<tr>
<td>Number of accessory cells per selected area (2)</td>
<td>50.3 ± 4.4</td>
<td>50.2 ± 5.0</td>
<td>53.8 ± 5.6</td>
<td>89.9 ± 5.9*</td>
<td>107.4 ± 8.6*</td>
<td>127.3 ± 6.9*</td>
<td>136.8 ± 5.2*</td>
</tr>
<tr>
<td>Number of immature cells per selected area (2)</td>
<td>11.8 ± 3.1</td>
<td>11.5 ± 2.2</td>
<td>12.4 ± 2.5</td>
<td>13.8 ± 5.2</td>
<td>22.5 ± 4.6*</td>
<td>27.5 ± 2.5*</td>
<td>33.8 ± 3.6*</td>
</tr>
<tr>
<td>Number of apoptotic cells per selected area (2)</td>
<td>6.7 ± 1.2</td>
<td>7.3 ± 1.4</td>
<td>7.4 ± 1.5</td>
<td>17.6 ± 3.8*</td>
<td>29.8 ± 4.0*</td>
<td>49.2 ± 6.5*</td>
<td>52.5 ± 3.0*</td>
</tr>
</tbody>
</table>

1- Number of apical pits on the rectangle area of filamental epithelium surface (2400 μm²)
2- Number of different subtypes of MRCs in 20 interlamellar regions
The proportion of cells within the four stages of the MRC life cycle also changed with salinity. The number of cells in mature, accessory, immature, or apoptotic stages quantified within 20 interlamellar regions, remained relatively consistent in fish exposed to 35-55 g/l salinity (Table 2.1). At 65-95 g/l salinity, the number of accessory and apoptotic cells significantly increased, and at 75 g/l the number of immature cells became significantly increased, while the number of mature cells decreased. Changes in cellular parameters are summarized in Table 2.1, and TEMs representing different stages of MRCs exposed to various salinities are presented in Figures 2.10-2.12. Finally, several cells from 95 g/l-exposed epithelia exhibited features that are considered signs of cellular necrosis, including electron-transparent vacuolated cytoplasm, swollen mitochondria possessing fragmented cristae, an irregularly organized tubular reticulum, and a nucleus with a low electron density (Fig. 2.12F-G).

Experiment IV: Oxygen Consumption Rate and Drinking Rate

Oxygen consumption rate ($\dot{M}O_2$) decreased with salinity. In fish acclimated to 95 g/l salinity, $\dot{M}O_2$ decreased 40.5 % relative to fish acclimated to seawater. Relative decreases in $\dot{M}O_2$ at 55 and 75 g/l salinity were 17.6 and 29.5% respectively (Fig. 2.13). Drinking rate remained constant at 35 and 55 g/l salinity, but was significantly increased at 75 g/l salinity (Fig. 2.14). Fish acclimated to 95 g/l for two weeks did not show an increased drinking rate, but these fish were in poor condition and means are based on n=3 due to mortality.
Figure 2.10. TEM showing the mitochondrial-rich cell ultrastructure from the filamental epithelium of tilapia exposed to 65 g/l salinity for 120 h.

A- Deep apical pit of a multicellular complex formed by interdigitated MRCs and ACs; arrowheads- short tight junctions, asterisks- projections of ACs.

B- Perinuclear cytoplasm of a mature MRC, with an extensively developed tubular reticulum.

C- MRC at the early stage of apoptosis.

D- Apoptotic MRC with a cleft nucleus and highly condensed cytoplasm: (Scale bars: 1μm).
Figure 2.11. TEM showing the mitochondrial-rich cell ultrastructure from the filamental epithelium of tilapia exposed to 75 g/l salinity for 120 h.

A- Nucleus and perinuclear cytoplasm of an apoptotic cell
B- Apical pit of multicellular complex that is partially covered by PC projections; asterisks-cyttoplasmic projections of accessory cells.
C- Apical pit that is completely separated from the ambient water by layer of PCs.
D- A multicellular complex without an apical pit; arrowheads indicate desmosomes joining MRCs and ACs.
E- MRC degrading by apoptosis.
F- MRC at the final stage of apoptosis (Scale bars: 1μm).
Figure 2.12. TEM showing the mitochondrial-rich cell ultrastructure from the filamental epithelium from tilapia exposed to 85 (A and B) and 95 g/l (C-G) salinity for 120 h.

A- Perinuclear cytoplasm of a mature chloride cell
B- MRC at the early stage of degradation by apoptosis
C- Nucleus and perinuclear cytoplasm of a mature cell
D- Multicellular complex with a shallow apical pit, formed by a mature cell at the early stage of apoptosis and AC projections
E- Apoptotic cell
F- Degenerated CCs with condensed cytoplasm, pycnotic nuclei, as well as distended and ruptured mitochondria
G- MRCs degrading by necrosis (Scale bars: 1 μm).
Figure 2.13. Oxygen consumption measured in tilapia hybrids acclimated for two weeks to 35, 55, 75, or 95 g/l salinity. Letters indicate significant differences (p<0.001, n=6).

Figure 2.14. Drinking rate measured in tilapia hybrids acclimated for two weeks to 35, 55, 75, or 95 g/l salinity. Letters indicate significant differences (p<0.001, n=6).
Discussion

‘California’ Mozambique tilapia were very saline tolerant, surviving up to 5 d in salinities as high as 95 g/l, which was similar to the salinity tolerance of pure Mozambique tilapia (Kültz and Onken, 1993; Stickney, 1986). When directly transferred from seawater, hybrid tilapia did not survive transfer to 85 g/l, but did survive transfer to 60 g/l, but experienced some osmoregulatory disturbances. Plasma osmolality, [Na⁺], and [Cl⁻] were restored to pre-transfer levels within 120 h.

When tilapia hybrids were gradually transferred to elevated salinity, with 120 h between transfers, they survived exposures as great as 65 g/l prior to showing any signs of osmoregulatory stress. Tilapia no longer maintained a steady state with respect to osmoregulation above 65 g/l salinity. Changes in gill morphology were most sensitive, and first appeared after 120 h of exposure to 55 g/l salinity, indicating that they may be good indicators of osmoregulatory stress in this species. More specifically, it was concluded that the number of apoptotic cells would best serve as an indicator of stress, due to their sizable increase immediately prior to other signs of osmoregulatory stress such as elevated plasma osmolality, [Na⁺], and [Cl⁻], and branchial Na⁺, K⁺-ATPase.

Experiment I: Seawater Acclimation of ‘California’ Mozambique Tilapia

Physiological, biochemical, and morphological data indicate that two weeks is sufficient for tilapia to become acclimated to seawater. Transfer from 20 to 35 g/l salinity resulted in increased plasma osmolality, [Na⁺], and [Cl⁻] 24 h following transfer (Fig. 2.1), but plasma osmolality decreased to a relatively constant level, near 340 mOsm/kg, when measured at 2 and 4 weeks. Although plasma [Na⁺] and [Cl⁻] remained
significantly elevated over the four week period, concentrations consistently remained near 180 and 160 mM, respectively, from two to four weeks. Hwang et al. (1989) found that seawater-acclimated Mozambique tilapia had a plasma osmolality and [Cl] of 343.8±17 mOsm/kg and 156±8.8 mM respectively, and Uchida et al. (2000) found that seawater acclimated tilapia had an osmolality of approximately 330 mOsm/kg. Furthermore, the general morphology and ultrastructure of the branchial epithelium of tilapia hybrids acclimated to 35 g/l salinity was similar to those previously observed in other seawater-acclimated species of tilapia (Cioni et al., 1991; Coleman et al., 1977; Hwang, 1987; van der Heijden et al., 1997; Wendelaar Bonga and van der Meij, 1989).

Experiment II: Mortality and Sub-lethal Effects of Direct Transfer to Increased Salinity

Survival was 100% following direct transfer from 35 to 60 g/l, but there were significant increases of plasma osmolality, [Cl] (Fig. 2.4), and Na⁺, K⁺-ATPase activity. Plasma [Na⁺] and [Cl] followed a similar pattern to plasma osmolality, which was significantly elevated at 24 h following transfer but returned to near pre-transfer values by 120 h (Fig. 2.4). The return of plasma osmolality to pre-transfer levels, in-spite of a greatly increased osmotic gradient, was most likely due to the nearly 260% increase in Na⁺, K⁺-ATPase activity that was observed 120 h following transfer. Kültz et al. (1992) also observed large increases in enzyme activity when transferring Mozambique tilapia from 10 g/l to 45, and 60 g/l salinity, respectively.
Experiment III: Sub-lethal Effects of Graded Transfer to Increased Salinity

The increase in salinity from 35 to 65 g/l represented a more than two-fold increase in the osmotic gradient between the water and the blood, yet no significant increases in plasma osmolality, [Na$^+$], or [Cl$^-$] (Fig. 2.5 and 2.6) were observed, nor was there an increase in Na$^+$, K$^+$-ATPase activity (Fig. 2.7). Kültz and Onken (1993) observed a dramatic reduction in mitochondrial-rich cell-accessory cell leaky junction conductance over the range of salinities from 35 to 60 g/l, and suggested that Mozambique tilapia reduce their whole body permeability in hypersaline water up to 60 g/l, which could offset the cost of osmoregulation in hypersaline water after a five week exposure.

The formation of interdigitations junctions observed in fish exposed to 55 g/l salinity may indicate that the fish are approaching the limit for effective osmoregulation via epithelial permeability reduction, and that a more traditional mechanism of salt excretion, such as an increase in Na$^+$, K$^+$-ATPase activity or drinking rate, is needed (Laurent, 1984). A similar phenomenon was described in Mozambique tilapia after 5 days of acclimation to full-strength seawater (Wendelaar Bonga and van der Meij, 1989). Another possible explanation for the lack of change in plasma osmolality, [Na$^+$], and [Cl$^-$] could be that the measured level of Na$^+$, K$^+$-ATPase activity is sufficient to deal with environmental salinities up to 65 g/l. Furthermore, in spite of the lack of change in gut Na$^+$, K$^+$-ATPase, the role of the gut in water absorption should not be ruled out; drinking rate was unchanged between 35 and 55 g/l, but increases in water absorption rate still may have occurred.
At exposure salinities greater than 65 g/l, there were significant changes in physiological, biochemical, and morphological indicators of osmoregulatory stress, which were probably indicative of osmoregulatory failure at the highest salinities. Plasma osmolality became significantly elevated, relative to seawater, 3 h following transfer to 85 and 95 g/l salinity and at 24 and 120 h following transfer to 75-95 g/l salinity, and plasma [Na\(^+\)] and [Cl\(^-\)] followed a similar trend (Fig. 2.5 and 2.6). The increased osmolality and ion levels coincided with a dramatic increase in Na\(^+\), K\(^+\)-ATPase activity at 75 and 85 g/l (Fig 2.7), which was a good indication of an osmoregulatory challenge.

Exposure salinities greater than 65 g/l increased the turnover rate of branchial MRCs. Changes in physiological and biochemical parameters in fish exposed to 65-95 g/l were associated with wide scale morphological alterations along the branchial epithelium. In 65 g/l-exposed fish, the number of accessory cells and apoptotic cells increased nearly two-fold and three-fold, respectively, but the number of mature cells was similar to fish exposed to 35-55 g/l salinity. Exposure to 75-95 g/l salinity resulted in dramatic changes in the ratio between different subtypes of MRCs. Mature cells constituted only 11% of the total number of cells in 75g/l-exposed fish, and only 5% in 95g/l-exposed fish; in contrast, mature cells constituted 32-36% of all MRCs in fish exposed to 35-55g/l salinity (Table 2.1). Similar results observed in freshwater-acclimated tilapia, exposed to a number of stressors (e.g. high salinity, low pH, or cadmium), were attributed to an increased turnover of MRCs due to rapid aging (van der Heijden et al., 1997; Wendelaar Bonga and van der Meij, 1989).
TEMs of the tilapia epithelium exposed to 75 g/l in the current study revealed partial and completely covered apical pits (Fig. 2.11B and C), which most likely occurred at other high salinities as well. It is unclear to what end tilapia in hypersaline water are using a freshwater acclimation strategy, but it may represent an attempt to seal off MRC leaky junctions to prevent further gain of salts or loss of water to the external environment. A more detailed investigation into this phenomenon would certainly prove interesting.

Fish exposed to salinities above 65 g/l face a further osmoregulatory challenge, as the branchial epithelium may have a drastically reduced capacity for ion transport. It has been previously shown that the ion-transporting capacity of the epithelium is dependent on the number of mature cells rather than total number (Kültz et al., 1992). Functional activity of accessory and immature cells should be lower relative to mature cells, due to their poorly-developed tubular reticulum and a limited number of mitochondria. Furthermore, a very low ion-transporting capacity, if any, may be expected from degenerating cells, and mature cells occluded by pavement cells may be considered functionally silent. Significantly increased plasma osmolality, [Na$^+$], and [Cl$^-$] were observed in concert with a significantly decreased number of mature cells. Interestingly, Na$^+$, K$^+$-ATPase activity was highest at 75 and 85 g/l salinity (Fig. 2.7); the source of the increased enzyme activity in a largely degenerating epithelium is another point of interest for further study. It is also unclear what the increased turnover rate of MRCs meant with respect to survival of the animal, but it is not likely that fish could survive long exposure periods at salinities above 55-65 g/l, in particular at 95 g/l when signs of cellular necrosis in the epithelium became apparent.
Experiment IV: Oxygen Consumption Rate and Drinking Rate

Oxygen consumption rate ($\dot{M}O_2$) decreased with salinity, as much as 40.5% from 35 to 95 g/l (Fig. 2.13). Although the majority of previous work on the effects of salinity on $\dot{M}O_2$ has focused on changes during acclimation from freshwater to seawater (Farmer and Beamish, 1969; Morgan et al., 1997), Swanson (1998) observed a reduction in $\dot{M}O_2$ at 55 g/l salinity relative to 35 g/l in milkfish (*Chanos chanos*), and attributed it to a reduction in activity level, allowing for greater use of metabolic energy for osmoregulation. An alternative hypothesis was proposed by Haney and Nordlie (1997), when sheepshead minnow (*Cyprinodon variegatus*) where $\dot{M}O_2$ decreased nearly 33% over a range of salinities from 40 to 100 g/l. This decrease was comparable to that observed in the current study. Haney and Nordlie (1997) suggested that the drop in $\dot{M}O_2$ was related to a change in the permeability of the branchial epithelium, as described by Kültz and Onken (1993), as the reduction in branchial permeability would result in less oxygen diffusion into the animal. These hypotheses are not mutually exclusive, and it is possible that both play a role in tilapia hybrid osmoregulation.

Drinking rate was not significantly changed at salinities below 75 g/l (Fig. 2.14). The lack of an increase over this salinity range indicates that the tilapia were not losing excess water to the environment in spite of the increase from 35 to 55 g/l, and the large increase in osmotic gradient between the blood and the water. This was consistent with the hypotheses formed in experiment III that suggested a preventative strategy of osmoregulation in hypersaline water up to a salinity of 65 g/l via a reduction in branchial permeability.
Summary

The ‘California’ Mozambique tilapia had a similar level of salinity tolerance to pure Mozambique tilapia (Costa-Pierce and Riedel, 2000; Hwang et al., 1989; Kültz and Onken, 1993; Stickney, 1986; Uchida et al., 2000; van der Heijden et al., 1997). Morphology, in particular the number of apoptotic cells in the epithelium, appeared to be the most sensitive and dramatic indicator of osmoregulatory stress. Gill morphology remained relatively unchanged until fish were exposed to a salinity of 55 g/l, but plasma parameters did not change until fish were exposed to salinities greater than 65 g/l. To better visualize these changes, all variables were plotted on a scale of relative change, with values obtained at 35 g/l set to one (Fig. 2.15). This interpretation provides a good template for potential salinity tolerance modeling for this species when exposed to graded salinity increases. Whether or not the use of gill morphology as an osmoregulatory stress indicator is effective over longer durations of exposure to hypersalinity remains to be seen.

The final goal of this study was to gain insight into the salinity tolerance of California Mozambique tilapia, which has ecological relevance when the continual increase in salinity of the Salton Sea is considered. It may be concluded that with all other variables kept constant, these hybrids tolerated salinities as high as to 65 g/l and showed little to no change in osmoregulatory parameters, with the exception of the decreased $\Delta Q$. Furthermore, tilapia hybrids appeared to use a strategy involving a reduction in permeability during hypersaline exposure, which has been previously described by Kültz and Onken (1993). Above 65 g/l, changes in physiological, biochemical, and morphological indicators of osmoregulatory stress became apparent,
indicating a limit to salinity tolerance without upregulation of osmoregulatory mechanisms to levels greater than those observed in seawater tilapia. To further add to this model, the effects of other abiotic factors such as temperature or hypoxia, which have been observed to fluctuate in saline lakes such as the Salton Sea, need to be included; temperature in particular has been shown to greatly reduce osmoregulatory ability (Al Amoudi et al., 1996).
Figure 2.15. Indices of change relative to values measured in seawater-acclimated animals. SW values are set to one, * denotes absolute value significant differences from SW.
Chapter Summary

1) Tilapia exhibited two distinct responses to salinity increases, which were dependent on the ambient salinity. When transfers were conducted at salinities from SW to as high as 65 g/l (in 10 g/l increments), there were no signs of osmoregulatory stress (i.e.; increased plasma osmolality, [Na$^+$], or [Cl$^-$], or decreased muscle water content), as well as no change in drinking rate, branchial or intestinal NKA activity, or MRC turnover. Above the transition point of 60 g/l, MRC turnover was increased as was drinking rate and NKA activity. Although at 75 g/l and higher there were increases in plasma osmolality, [Na$^+$], and [Cl$^-$], muscle water did not change relative to SW-acclimated tilapia.

2) MRC apoptosis, indicative of an increased cell turnover rate, increased by as much as 8-fold during exposure to salinities from 65-95 g/l.

3) Whole animal oxygen consumption rate decreased with ambient salinity following 14 d of exposure where oxygen consumption rate was 40% lower at 95 g/l relative to 35 g/l.
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CHAPTER 3: METABOLIC SUPPRESSION IN RESPONSE TO HYPERSALINE EXPOSURE IN A MOZAMBIQUE TILAPIA HYBRID (*Oreochromis mossambicus* x *O. urolepis hornorum*) \(^2\)

**Introduction**

Teleost fishes in general are hypoosmotic relative to seawater (SW) and therefore lose water to the environment. To compensate, drinking rate is typically higher in SW-relative to freshwater (FW) acclimated fishes and the excess ions are excreted by branchial mitochondrial-rich cells (MRCs). Mozambique tilapia (*Oreochromis mossambicus*) are very good models of teleost euryhalinity. Previously, it was found that tilapia were able to survive direct transfer from FW to 20 g/l salinity, but not a direct FW to SW transfer (Hwang et al., 1989). In switching from a hyper- to a hypoosmoregulatory strategy, tilapia experienced a ‘crisis’ period from 6 to 12 h following transfer, in which they must survive near-lethal levels of dehydration (Hwang et al., 1989). The crisis period was followed by a period of stabilization from 12-24 hours, when plasma ion levels returned to control values. The beginning of stabilization was correlated with the observation of leaky paracellular junctions, which are necessary for Na\(^+\) excretion (Hwang et al., 1989; Hwang, 1987). Using the opercular membrane as a model for the branchial epithelium, Foskett et al. (1981) showed that MRC number increased during the first 3 d post-transfer to SW, at which time MRCs begin to hypertrophy.

While much is known regarding physiological responses during transfer from FW to SW, only a few studies have investigated the effects of exposure to salinities greater than SW (Kültz and Onken, 1993; Sardella et al., 2004b; Stickney, 1986). Using the

\(^2\) A version of this chapter has been submitted for publication to the Journal of Experimental Biology
opercular epithelium, Kültz and Onken (1993) examined the electrophysiological effects of a FW to SW transfer, as well as exposure to hypersaline conditions in tilapia (i.e., salinities greater than SW), and showed that tilapia have a different osmoregulatory strategy to deal with hypersaline environments compared to what was observed during FW-SW acclimation. After a five week exposure to 60 g/l salinity, epithelial permeability relative to SW acclimated fish was reduced. Although the number of MRCs was greater at high salinity, the conductances of both the transcellular and paracellular pathways were greatly reduced (Kültz and Onken, 1993). The results of Kültz and Onken (1993) indicated that tilapia were able to defend against osmotic water loss via reduction in epithelial permeability while a constant Na\(^+\) and Cl\(^-\) excretion rate was maintained.

The in vivo salinity tolerance of California Mozambique tilapia (Oreochromis mossambicus x O. urolepis hornorum) has been assessed during progressive increases in salinity by Sardella et al. (2004b). Over the range of salinities from 35 to 55 g/l tilapia maintained plasma osmolality, [Na\(^+\)], [Cl\(^-\)] and the water content of white muscle despite no significant changes in branchial Na\(^+\), K\(^+\)-ATPase (NKA) activity, drinking rate, or MRC turnover. At salinities of 65 g/l and greater, tilapia responded to increased salinity with increased NKA activity, drinking rate, and MRC turnover; however, plasma osmolality and ion levels were also increased at these high salinities (Sardella et al., 2004b). Above the transition point of 60 g/l, the response of tilapia was typical of teleost fish when faced with an osmotic challenge (Sardella et al., 2004b).

In addition to the observation of two distinct osmoregulatory responses during exposure to progressively higher salinity, tilapia showed a steady decrease in whole animal oxygen consumption rate (\(\dot{M}O_2\)) with salinity increase (Sardella et al., 2004b).
Decreases similar to these have also been measured in other euryhaline fish species (Dead Sea killifish *Aphinius dispar* (Plaut, 2000); sheepshead minnow *Cyprinodon variegatus* (Haney and Nordlie, 1997); milkfish *Chanos chanos* (Swanson, 1998)). It has been hypothesized that a reduction in metabolic rate during hypersaline exposure may be attributed to reduced spontaneous activity of individual fish and/or reductions in gill permeability in defense against an extreme osmotic gradient. Furthermore, it has been suggested that reductions such as these are indicative of a “last ditch effort” by fish in order to survive a deleterious environment, and that these reductions would reflect an upper limit for salinity tolerance (Plaut, 2000). Our previous study showed a reduction in whole animal \( \dot{M}O_2 \) as high as 40% between fish acclimated to 95 g/l relative to 35 g/l salinity for 14 d (Sardella et al., 2004b); however, the time course over which \( \dot{M}O_2 \) is reduced and the physiological basis for the reduction remain unknown.

The purpose of this study was to investigate the time course and physiological basis for salinity-induced decreases in whole animal \( \dot{M}O_2 \), and to investigate how the length of exposure affects the transition between the two responses defined previously for this species. FW-reared tilapia hybrids were exposed in 15 g/l steps to 15, 30, 45, 60, and 75 g/l salinity, with samples taken at 1, 5, 14, and 28 d; plasma osmolality and muscle water content, as well as branchial NKA activity were measured in order to assess the effects of hypersaline exposure on osmoregulation. In addition, we measured ATPase activities in muscle, liver, kidney, gut, and brain in order to correlate the activities of these tissues with \( \dot{M}O_2 \), and test the hypothesis that salinity-based reductions in \( \dot{M}O_2 \) have a biochemical basis in this species.
Materials and Methods

Fish

California Mozambique tilapia (*Oreochromis mossambicus* x *O. urolepis hornorum*) were generously donated by Pacific Aquafarms in Niland, CA, USA. Fish were reared in FW, and transported to the University of British Columbia, where all experiments were conducted. Tilapia averaging 6.2 ± 1.9 g (28/tank) were acclimated to 15, 30, 45, 60, and 75 g/l salinity in a stepwise fashion. Salinities were increased by one-half volume water changes every 7 d, until reaching the target salinity, at which time fish were held for 28 d; water quality was maintained in all tanks by use of mechanical, chemical and biological filtration. During salinity acclimation and throughout the time course of exposure, temperature was held at 25°C, and fish were fed *ad libitum*, with feeding withheld 24 h prior to measurement of oxygen consumption rate and/or sampling. Tanks were 60 l in volume, and held up to 28 fish initially with the number decreasing by seven with each sampling. The tilapia used for measurements of \( \dot{M}O_2 \) were held separately from those used for tissue samples in 40 l tanks at the respective salinities, and repeatedly used to measure \( \dot{M}O_2 \) at each time point. Salinity was manipulated using Instant Ocean Synthetic Sea Salt in dechlorinated water, and measured by a light refractometer.

Oxygen Consumption

Once fish were exposed to their target salinity (15, 30, 45, 60, or 75 g/l), oxygen consumption rate (\( \dot{M}O_2 \)) was measured at 1, 5, 14, and 28 days. Tilapia were transferred to individual dark respirometry chambers (~360 ml), at the respective salinities, with
gravity-fed water flow, and allowed to acclimate for 12-14 hours (overnight) before measurements of $\dot{M}O_2$ were taken; using killifish (*Fundulus heteroclitus*) (Kidder III et al., 2006) showed that exploratory movements typically ceased and that oxygen uptake stabilized after 12 h. Following acclimation, water flow was stopped, and the decrease in water $O_2$ content within individual chambers was continuously measured for approximately 25 minutes (flow was re-initiated when water $O_2$ level reached 70-75% saturation). The decrease in water $O_2$ content was measured using an Ocean Optics FOXY $O_2$ probe and Ocean Optics software (Ocean Optics Inc., Dunedin, FL, USA). The oxygen electrode was calibrated to air (100%) and $N_2$ flow (0%) prior to and following each measurement. After $\dot{M}O_2$ measurements, the mass of the fish and the volume of water in the chamber were recorded. Using the slope of the decrease in %$O_2$ over time, the weight of the fish, the water volume of the chamber, and $O_2$ solubility coefficients ($\alpha$), adjusted for temperature and salinity, $\dot{M}O_2$ was calculated as mg $O_2$/kg/h.

Fish Sampling

Seven tilapia were sacrificed by a lethal dose of MS-222 at 1, 5, 14, and 28 days after reaching each target salinity. The caudal peduncle was severed and blood was collected into heparinized capillary tubes, which were then centrifuged in a Damon IEC MB microhematocrit centrifuge. Hematocrit (Hct) was recorded in duplicate or triplicate, depending on available blood volume, and plasma was expelled into microcentrifuge tubes for measurement of plasma osmolality, which was measured using a WESCOR 1500 vapor pressure osmometer. Approximately 0.2 g of left dorsal epaxial muscle was
removed; the muscle was separated from the skin, quickly rinsed, and patted dry. The muscle was placed into pre-weighed aluminum foil and dried at 70°C for 96 h to determine the % muscle water. The gills, brain, liver, intestine, kidney, and a similarly-sized piece of epaxial muscle from the right side of the fish were frozen in liquid N₂ for later analyses.

Enzyme Assays

All tissues were stored at -80°C until enzyme assays were conducted. Gill, brain, muscle, gut, kidney, and liver were homogenized in approximately 1 ml of SEID buffer (250 mM sucrose, 10 mM EDTA-Na₂, 50 mM imidazole, pH 7.3, 0.05% deoxycholic acid). Na⁺, K⁺-ATPase activity was determined as the ouabain-inhibited fraction of total oxidation of NADH as described by (McCormick, 1993) and expressed as μMol of ADP/h/μg total protein. In addition, we measured total tissue ATPase activity as the ouabain uninhibited activity curve, which was also standardized to total protein content of the homogenate, determined using the Biuret method. We were unable to detect ouabain-inhibitable fractions of NKA in tilapia muscle, and thus could not attain accurate estimates of muscle NKA activity. Furthermore, while ouabain-uninhibitable fractions in this tissue were very large at all salinities, we did not calculate total muscle ATPase due to the uncertainty of the amount of contractile apparatus that may have remained in the homogenate. In hepatic tissue, once again, the fraction of total ATPase activity that was ouabain-inhibitable was too small to distinguish from variation within the assay, however, we have standardized the ouabain-uninhibitable fraction to total protein in the homogenate, and presented it as total hepatic ATPase activity. Lastly, hepatic PEPCK
activity was extremely low in all salinities at all times; the level of PEPCK activity was below the detection level for this assay, which was determined to be approximately 0.267 μmol/kg/h using liver tissue from juvenile Chinook salmon (Oncorhynchus tshawytscha).

Statistical Analyses

A two-way analysis of variance (ANOVA), followed by a post-hoc Holm-Sidak test, was used to assess the effects of time and salinity on $\dot{M}O_2$, plasma osmolality, muscle water content, and branchial NKA activity. ATPase activities (excluding muscle) were correlated with $\dot{M}O_2$ using forward-stepwise multiple linear regression; these analyses were conducted for the data set as a whole and also broken down by day. The regression provided a model equation, including only those variables that significantly correlated with metabolic rate. All statistical analyses were performed using SigmaStat version 3.0, and plots were made using SigmaPlot version 8.0. Unless otherwise indicated, values presented are mean±SE.

Results

There was no mortality observed throughout this study. All fish appeared active and consistently ate all the food presented to them regardless of salinity.

Oxygen Consumption

There was a significant effect of salinity on $\dot{M}O_2$ ($p < 0.001$), as well as a significant interaction between time of exposure and salinity ($p < 0.001$). The overall trend was for $\dot{M}O_2$ to decrease with salinity at all time points except 28 d, when there were no significant differences in $\dot{M}O_2$ among the salinity groups (Fig. 3.1). Although


\( \dot{M}O_2 \) was significantly decreased at 1 and 5 d following exposure to 60 g/l salinity, the greatest reductions in metabolic rate were observed following 14 d of salinity exposure, where 60 g/l-acclimated fish had a significantly reduced \( \dot{M}O_2 \) relative to 15 and 30 g/l fish (35 and 32% reduced, respectively). Additionally, the highest \( \dot{M}O_2 \) was measured in tilapia exposed to 15 g/l salinity for 5 d; this value was significantly greater than all other values at this time.

**Osmoregulatory Variables**

Plasma osmolality was largely unchanged at all times in fish exposed to 15, 30, 45, and 60 g/l salinity (Fig 3.2); there were some significant differences among salinities at a given time, but they were very small relative to those observed at 75 g/l. At all time points, tilapia in 75 g/l water had a significantly higher plasma osmolality relative to all other salinities. Two-way ANOVA revealed a significant effect of time of exposure, salinity, and time/salinity interaction (all p values < 0.001).

Branchial NKA activity was also significantly affected by salinity, time of exposure and salinity/time interaction (all p values <0.001). The greatest NKA activity levels were observed in fish exposed to 75 g/l salinity (Fig. 3.3); these values were significantly greater than those observed at 60 g/l at all time points except 28 d, where values were similar. There were other slight but significant differences as detected by two-way ANOVA, however, as with plasma osmolality, these were minor in comparison to the large increases at 60 or 75 g/l (Fig. 3.3). Salinity also had a significant effect on the level of intestinal NKA activity, with a significant increase measured in 75 g/l-acclimated fish relative to those acclimated to 15-60 g/l (data not shown).
Figure 3.1. A) Changes in whole-animal oxygen consumption rate over time in California Mozambique tilapia (*Oreochromis mossambicus* x *O. urolepis hornorum*) following transfer to various salinities; (●) represents a value from FW-acclimated animals for comparison, but is not included in statistical analyses. Symbols indicate mean±SE; n=7. Letters that differ indicate differences between values from different salinities at a common time, while numbers that differ indicate differences with time within a given salinity (p<0.001; n=7). B) A three dimensional plot of oxygen consumption rate as a function of time and salinity.
Figure 3.2. Changes in plasma osmolality over time in California Mozambique tilapia (*Oreochromis mossambicus* x *O. urolepis hornorum*) following transfer to various salinities; (●) represents a value from FW-acclimated animals for comparison, but is not included in statistical analyses. See figure 3.1 for details. B) A three dimensional plot of plasma osmolality as a function of time and salinity.
Figure 3.3. Changes in branchial Na\(^+\), K\(^+\)-ATPase (NKA) activity over time in California Mozambique tilapia (*Oreochromis mossambicus* x *O. urolepis hornorum*) following transfer to various salinities; (●) represents a value from FW-acclimated animals for comparison, but is not included in statistical analyses. See figure 3.1 for details. B) A three dimensional plot of branchial NKA activity as a function of time and salinity.
Finally, muscle water content and muscle $[\text{Na}^+]$ showed no significant changes throughout all treatments (pooled values: 79.4±0.5% and 0.17±0.01 mmol/g dry tissue, respectively). Regression analyses showed no significant relationship between plasma osmolality and muscle water content at any of the sampling times (Fig. 3.4).

Enzyme Activities

The forward-stepwise multiple regression model for the data set as a whole indicated that tilapia whole animal $\dot{M}_O_2$ was best described as a function of branchial NKA activity (negative correlation) and total hepatic ATPase activity (positive correlation) under these conditions. However, as the degree of change in metabolic rate differed depending on time following transfer to salinity, regression models conducted on a by-day basis proved more interesting. Our analyses showed that no variables correlated with $\dot{M}_O_2$ at 1 and 28 d following transfer. The regression model showed that the activity of brain NKA best described the changes in $\dot{M}_O_2$ ($p<0.01$, $r^2=0.944$; Fig. 3.5) after 5 d of exposure. As was observed with the data set as a whole, $\dot{M}_O_2$ was best correlated with gill NKA activity and total hepatic ATPase activity (Fig. 3.6) 14 d following transfer ($p=0.021$, $r^2=0.957$ and $p=0.02$, $r^2=0.984$, respectively).
Figure 3.4. Regression analyses of muscle water content as a function of plasma osmolality; graphs represent correlations at 1, 5, 14, and 28 d following transfer to salinity (see individual graphs for statistical values). No regressions were significant except at 14 d where the slope was positive, regardless of a trend toward negativity at 1 and 5 d following transfer; plasma osmolality did not significantly affect muscle water content at any time. Symbols represent individual data points, and dashed lines represent 95% CI.
Figure 3.5. Whole animal oxygen consumption plotted as a function of brain Na\(^+\), K\(^+\)-ATPase activity after 5 d of exposure to salinities ranging from 15 to 75 g/l. Brain activity and oxygen consumption were highly correlated at this time interval (p<0.01; \(r^2=0.944\)). Symbols represent mean±SE, and dashed lines represent 95% CI, n=7.

Figure 3.6. Whole animal oxygen consumption plotted as a function of gill NKA activity and liver total ATPase activity after 14 d of exposure to salinities ranging from 15 to 60 g/l. Gill NKA was negatively correlated with oxygen consumption rate (p=0.021, \(r^2=0.975\)) and liver ATPase was highly positively correlated with oxygen consumption rate at this time (p=0.02, \(r^2=0.984\)); see Fig. 3.5 for details.
Discussion

This study is the first to report a tissue-level metabolic suppression during exposure to elevated salinity. Previous studies have demonstrated reductions in $\dot{M}O_2$ in euryhaline species during exposure to hypersalinity; however in this study we have shown that the decrease in tilapia $\dot{M}O_2$ has a strong positive correlation with total hepatic ATPase activity. In addition, the results have provided further support for the idea that this species of tilapia exhibits two acclimatory responses during exposure to increased salinities (Sardella et al. 2004b). Biphasic responses to increased salinity have also been observed in sheepshead minnow (*Cyprinodon variegatus*; Nordlie, 1984) and sailfin mollies (*Poecilia latipinna*; Gonzalez et al., 2005). The transition point for switching between the two patterns was observed at salinity of approximately 60 g/l when temperature was held at 25°C, and did not appear to be significantly affected by exposure duration (i.e.: 5 d to 28 d). It should be noted however, that deviations of ± 10°C can dramatically lower this transition point (Sardella et al., 2004a). There was a clear reduction in $\dot{M}O_2$ across the range of salinities associated with the "Phase I" acclimatory response (from 15 to 60 g/l). Metabolic reductions such as this have previously been observed in this (Fig. 3.7; Sardella et al., 2004b), and other species (Haney and Nordlie, 1997; Plaut, 2000; Swanson, 1998). We have also shown that the reductions in $\dot{M}O_2$ are not only dependent on salinity, but also time of exposure, as there were no differences in $\dot{M}O_2$ across the salinity range by 28 d following transfer (Fig. 3.1).
Figure 3.7. Whole animal oxygen consumption plotted as a function of ambient salinity at 14 d in California Mozambique tilapia (Oreochromis mossambicus x O. urolepis hornorum); data is pooled from the current study (○) as well as Sardella et al., (2004b) (●).
The Dual Acclimation Responses

At salinities above the transition point of 60 g/l, drinking rate, branchial and intestinal NKA and MRC turnover increase, as do signs of osmoregulatory stress (plasma osmolality, [Na\(^{+}\]), and [Cl\(^{-}\)]); this response has been observed in this study and in previous work with this species and was referred to as the "active" response to salinity increase (Sardella et al., 2004b). In the current study, tilapia exposed to 75 g/l salinity for 1 d showed a dramatic increase in plasma osmolality, but recovered to an elevated but stable level at 5, 14, and 28 d (Fig. 3.2). The partial recovery and stabilization of plasma osmolality was likely due to the large increase in intestinal and branchial NKA activity, which remained high throughout the remainder of the exposure.

Maintenance of Muscle Water Content

Interestingly, at no time during this or our previous study, was there a change in muscle water content or muscle [Na\(^{+}\)]. When broken down by day, it was apparent that when increases in plasma osmolality did occur (1 and 5 d primarily at 75 g/l salinity) they were not enough to perturb muscle water content despite increasing by as much as 50%. Maintenance of muscle water was also observed during transfers to salinities as high as 95 g/l by (Sardella et al., 2004b). The maintenance of muscle water content in this species in the face of such high plasma osmolalities was remarkable. In salmonids for example, there was a strong negative correlation between plasma ionic status and muscle water content over a much smaller range of changes in plasma osmolality (Brauner et al., 1994; Brauner et al., 1992). Furthermore, sailfin mollies, a species with a high salinity tolerance similar to tilapia, were unable to maintain muscle water content in salinities...
above 75 g/l (Gonzalez et al., 2005). Clearly the ability of this species to avoid muscle water loss in spite of increased plasma osmolality is worthy of further investigation.

Tissue Level Changes associated with Reduced Oxygen Uptake

Activity levels of intestinal and renal NKA remained essentially constant across all salinities and time points (27.5±0.8 and 32.1±1.3 μmol ADP/h/μg Pr., respectively), and were excluded from the regression model representing both the entire data set and by-day analyses. While brain NKA activity was not correlated with changes in metabolic rate in the complete data set, at 5 d following transfer, the correlation with $\dot{M}O_2$ was significant (Fig 3.4). Furthermore, both brain activity and $\dot{M}O_2$ were very high in the 15 g/l exposed fish at 5 d, which may have reflected the energy demand for switching between a hyper- and a hypoosmoregulatory strategy. It was previously found that tilapia can take up to 7 d to fully acclimate to SW with respect to MRC function (Hwang, 1987). The elevated $\dot{M}O_2$ and brain activity observed in this study may have reflected the costs of gill epithelium restructuring, and perhaps the endocrine role of the pituitary in SW acclimation (Borski et al., 1994; McCormick, 2001; Seidelin and Madsen, 1999).

Possibly the most interesting finding of this study was the very strong correlation between total liver ATPase and whole animal $\dot{M}O_2$ at 14 d following transfer, in particularly within the from 15 to 60 g/l salinity range (Fig. 3.5). Regression models indicated that $\dot{M}O_2$ is best described by both liver ATPase as well as gill NKA activity, although the correlation between $\dot{M}O_2$ and liver ATPase was positive. After 14 d of exposure to the salinity range from 15 to 60 g/l, the ability of this tilapia species to reduce $\dot{M}O_2$ correlated proportionally with the decrease in liver ATPase activity. We do not
believe the reduction in $\dot{M}O_2$ across this range is indicative of a last ditch effort to survive, as the greatest reduction in $\dot{M}O_2$ was observed at 14 d following transfer when plasma osmolality, muscle water content, and branchial NKA activity were essentially identical to those of SW acclimated animals. The decrease in liver ATPase activity indicates that there was a biochemical/physiological basis for the salinity-based reduction of $\dot{M}O_2$. In addition, the negative correlation between gill NKA activity and $\dot{M}O_2$ was interesting in that it demonstrated osmoregulation at the gill may not be as energetically expensive as has been proposed by some (Rao, 1968), and may be more in line with the values proposed by others (Kirschner, 1995; Morgan and Iwama, 1999). The large increase in $\dot{M}O_2$ and high brain NKA activity 5 d following transfer from FW to 15 g/l coincides with the timing of branchial epithelium rearrangement associated with switching from a hyper- to hypoosmoregulatory mechanism, indicating that this process may be energetically expensive.

A Potential Mechanism behind the “Phase I” Response to Salinity Increase

When ambient salinity ranged from 15 to 60 g/l, tilapia were able to maintain osmotic balance in the plasma and muscle compartments with no observable upregulation of osmoregulatory mechanisms relative to those measured in SW. Sardella et al. (2004b) showed that drinking rate in this species did not change relative to SW fish in salinities below 75 g/l, indicating no increase in osmotic water loss relative to that experienced by SW-acclimated fish. The decrease in $\dot{M}O_2$ observed in this and previous studies may be the basis for how tilapia accomplished this. Decreased gill blood and water flow, associated with a reduction in metabolic rate, would reduce the potential for osmotic
water loss and diffusive ion gains across the gill. During this time (up to at least 14 d),
the conductance of the gill epithelium could be altered (as described by Kültz and Jurss,
1993) such that by 28 d, $\dot{M}$O$_2$ can return to pre-transfer values without negatively
affecting osmoregulatory status. It appeared that this mechanism alone may be sufficient
for tolerance of salinities as high as 60 g/l. The transition point for switching between the
two acclimation strategies that was apparent in this and the previous study (Sardella et al.,
2004b) may represent an ambient environment in which the osmotic gradient was too
great, and the benefits to osmoregulation associated with metabolic suppression are not
sufficient, making upregulation of osmoregulatory capacity necessary. Further
investigation into metabolic suppression as a salinity tolerance mechanism should prove
interesting.
Chapter Summary

1) This was the first evidence of metabolic suppression in response to a salinity challenge, which may play a role in the high tolerance of this species.

2) The two distinct responses to increasing salinity in this species were maintained for as long as 28 d following transfer.

3) $\hat{M}O_2$ decreased proportionally with ambient salinity and was correlated with a reduction in total hepatic ATPase activity indicating a biochemical basis for the salinity induced reduction in metabolic rate.

4) In spite of dramatically increased branchial NKA activity with salinity up to 75 g/l, $\hat{M}O_2$ progressively decreased, indicating that the energetic costs associated with increased gill NKA capacity are small in comparison with the other whole animal responses observed.

5) $\hat{M}O_2$ was highest during transition from hyper- to hypoosmoregulatory regulation, which may be indicative of a high cost for epithelial restructuring.
References


PART TWO: THE EFFECTS OF TEMPERATURE ON TILAPIA
OSMOREGULATORY CAPACITY
CHAPTER 4: THE EFFECT OF TEMPERATURE ON JUVENILE MOZAMBIQUE TILAPIA HYBRIDS (Oreochromis mossambicus x O. urolepis hornorum) EXPOSED TO FULL-STRENGTH AND HYPERSALINE SEAWATER\(^3\).

**Introduction**

Mozambique tilapia (*Oreochromis mossambicus*) are one of the best studied models of euryhalinity among fishes, and have provided insight into the physiological challenges and solutions associated with the transition from freshwater to seawater (Hwang et al. 1989, van der Heijden et al. 1997, Morgan et al. 1997, Lee et al. 1998, Uchida et al. 2000, Seale et al. 2002, Weng et al. 2002). Furthermore, Mozambique tilapia have been reported to withstand extremely high salinities, and have been regarded as one of the most saline tolerant teleosts (Stickney, 1986). Surprisingly, however, there are very few studies investigating the physiological mechanisms associated with transfer to salinities greater than seawater (Kültz and Jurss 1993, Kültz and Onken 1993). It has been also reported that temperature influences the osmoregulatory ability of fishes, where a reduction in temperature below an optimal value induced greater osmoregulatory disturbances than a similar elevation in temperature (Al Amoudi et al. 1996, Handeland et al. 2000, Staurnes et al. 2001, Imsland et al. 2003, Metz et al. 2003). Mozambique tilapia are relatively eurythermal, but have a lower lethal temperature that is relatively high (10-15°C) compared to other species (Costa-Pierce and Riedel 2000). It is likely that temperature plays a crucial role in salinity tolerance of Mozambique tilapia, however this has not been explored in detail.

\(^3\) This chapter was modified from a previously published article; Sardella, B. A., J. Cooper, R. J. Gonzalez, and C. J. Brauner in Comparative Biochemistry and Physiology A 137:621-629 (2004).
California Mozambique tilapia (*Oreochromis mossambicus* x *O. urolepis hornorum*) currently resides in southern California (Costa-Pierce and Doyle 1997). Living within drainage ditches, creeks, aquaculture farms, and the Salton Sea, this hybrid tilapia inhabits a large range of temperatures (10-35 °C) and is exposed to salinities exceeding that of seawater (Costa-Pierce and Doyle 1997). The objectives of this study were to investigate the interactive effects of temperature and salinity on the California Mozambique tilapia. Tilapia that had been previously acclimated to 35 g/l salinity at 25°C, were directly transferred to 35, 43, 51, or 60 g/l salinity, at 15, 25, or 35°C, for 24 h. The effects of transfer were determined by monitoring mortality and measuring sublethal indicators of osmoregulatory stress. In salmonids, 24 h seawater challenge tests are routinely used to assess the hypoosmoregulatory ability of smolts prior to their release from hatcheries (Clarke and Blackburn 1977, Wedemeyer et al. 1980, Blackburn and Clarke 1987). In these challenges, fish are directly transferred from freshwater to full strength seawater, and following 24h, plasma \([Na^+]\) and/or \([Cl^-]\) are measured and provided the values fall within a pre-determined range, the smolts are considered pre-acclimated for seawater entry. Clarke and Blackburn (1977) have shown that salmonids that performed well in the 24 h seawater challenge test had the greatest growth rates once they were transferred to seawater. In addition, Hwang et al. (1989) described the first 24 h following a salinity transfer as the most crucial in Mozambique tilapia, as they must survive high levels of dehydration. The period from 12 to 24 h was determined to be the beginning of a ‘stabilization period’ in which salt excretion mechanisms began to function. Our modified 24 h salinity challenge test may be very useful in quantifying the threshold and mechanisms of salinity tolerance of the California Mozambique tilapia, and
provide a first step in understanding how environmental conditions such as salinity and temperature may limit the distribution of this fish.

Finally, we investigated the effect of acclimation to 15, 25, and 35°C at 35 g/l for 2 weeks on branchial Na⁺, K⁺-ATPase activity to determine the degree to which changes in branchial enzyme activity may compensate for the effects of temperature on hypoosmoregulatory ability. Gill tissue taken from seawater fish at these three temperatures was assayed at 15, 25, and 35°C. The effect of assay temperature on Na⁺, K⁺-ATPase activity was investigated as the best approximation of the effects of a sudden temperature change on this enzyme in vivo in a previously temperature-seawater acclimated fish.

Materials and Methods

Fish

Pacific Aquafarms in Niland, California donated 300 juvenile tilapia hybrids (Oreochromis mossambicus x O. urolepis hornorum) for use in this study. Hybrids were acclimated to 25°C seawater (35 g/l) following three salinity transfer increments (0-10 g/l, 10-20 g/l, and 20-35 g/l) in a 440 liter filtered, aerated, tank with four days allowed for acclimation at each salinity. Fish were then left for two weeks at full strength seawater (35 g/l). Tilapia averaged 32.68 ± 0.68 g in mass, and were fed commercial trout food daily; feed was withheld 24 h prior to sampling. Seawater was prepared using Instant Ocean synthetic sea salt in dechlorinated tap water, and salinity was measured using a light refractometer.
Experiment I: 24 h Hypersaline Challenges

Salinity challenges were conducted in 60 l glass aquaria, static systems with aeration and filtration with Siporex biofilters were used in all treatments. Fish were subjected to a 24 h salinity challenge by direct transfer of seven fish at a time from the large holding tank to the smaller tanks containing water with 35, 43, 51, or 60 g/l salinity, at 15, 25, or 35°C, which yielded a total of 12 treatments. Additional salinity transfers to 47 g/l were included, at 15 and 25°C, based upon the results obtained from other treatments. Within each treatment, surviving fish were terminally sampled following 24 h of exposure (max. n=7).

Prior to sampling, tilapia were anesthetized with Benzocaine, dissolved in 3 ml ethanol, and diluted to a final concentration of 0.7 g/l. Fish were then rinsed with distilled H₂O, patted dry, and weighed, prior to severing of the caudal peduncle. Blood was collected into heparinized microhematocrit capillary tubes, and centrifuged at 11,500 RCF for three minutes in a Damon IEC MB microhematocrit centrifuge. Hematocrit (Hct) was recorded in duplicate or triplicate depending on available blood volume and plasma was expelled into heparinized microcentrifuge tubes and frozen at -80°C. Whole blood total hemoglobin concentration ([Hb]) was measured using a Sigma total hemoglobin assay kit, with absorbance measured at 540nm; [Hb] was then converted to mean cell hemoglobin concentration (MCHC), expressed as [Hb]/(Hct/100).

Approximately 1.5 g of the left dorsal epaxial muscle was removed to determine muscle water content. Muscle tissue was placed into pre-weighed, plastic scintillation vials and weighed prior to, and following drying to constant weight for 72-96 hours at 70°C.
Plasma osmolality was measured using a Wescor 5500 vapor pressure osmometer, and expressed as mOsm/kg H2O. Plasma [Cl⁻] was measured using the colorimetric mercuric thiocyanate method (Zall et al. 1956), and plasma [Na⁺] was measured with an atomic absorption spectrophotometer (Perkin Elmer model 3100 A).

The effect of temperature acclimation on branchial Na⁺, K⁺-ATPase in seawater-exposed tilapia

Groups of seven 35 g/l-acclimated fish were transferred to 15, 25, or 35°C water for two weeks. Fish were then anesthetized as described above and gills removed, frozen on dry ice, and stored at -80°C. Gill tissue was homogenized in SEI buffer (250 mM sucrose, 10 mM EDTA-Na₂, 50 mM imidazole, pH 7.3). Homogenates from fish at each acclimation temperature were assayed at 15, 25, and 35°C. Branchial Na⁺, K⁺-ATPase activity was determined using the method of Gibbs and Somero (1990) and expressed as μMol of ADP per hour per mg total protein (μMol/h/mg); protein was determined using the Biuret reagent.

Statistics

The interactive effects of temperature on Hct, MCHC, plasma osmolality, [Na⁺] and [Cl⁻], muscle water content, and Na⁺, K⁺-ATPase activity were analyzed using a two-way ANOVA, followed by post-hoc Bonferroni and Tukey HSD tests. Alpha values for significance were 0.05 in all statistical tests. ANOVAs were performed using SPSS version 10.0, and regressions were performed using Sigma Plot version 6.10, 2000 (SPSS Inc.).
Results

24 h hypersalinity challenges

Tilapia survived 24 h in all salinities at 25 and 35°C, however, at 15°C, mortality was 85.7 and 100% in the 51 and 60 g/l groups, respectively. Prior to death, fish appeared disoriented and inactive. In contrast, fish at 35°C exhibited high activity and ventilation at all salinities. Because mortality at 15°C increased from 0 to 85.7% between 43 and 51 g/l, follow-up salinity transfers to 47 g/l were included, at 15 and 25°C. All fish survived at both temperatures, indicating the onset of mortality at 15°C was between 47 and 51 g/l by this protocol.

There was significant interaction between temperature and salinity on plasma osmolality, [Na⁺], and [Cl⁻] in surviving fish (p < 0.001) (Fig. 4.1). A post hoc Bonferroni test revealed significant difference due to both salinity and temperature. As expected, plasma [Na⁺] exhibited qualitatively similar trends to osmolality, but plasma [Cl⁻] was largely unchanged, with the exception of fish transferred from 25 to 15°C in 35 g/l salinity (Fig. 4.1b and 4.1c). Due to mortality, plasma osmolality, [Na⁺], and [Cl⁻] for 15°C treatments at 51 and 60 g/l could not be measured. Osmolality at 35 g/l was significantly increased relative to 43 g/l (Fig. 4.1a). Muscle water content showed a small significant interaction between temperature and salinity (p = 0.047) (Fig. 4.2). Finally, no significant differences were observed in Hct or MCHC values (pooled values = 24.1±0.1 and 2.84±0.12 respectively; data not shown).
Figure 4.1. The effect of simultaneous salinity (35, 43, 51, and 60g/l) and temperature (15, 25, and 35°C) 24 h following transfer on a) plasma osmolality, b) plasma chloride, and c) plasma sodium in tilapia hybrids. Roman numerals (I, II, III) indicate significant effects of temperature, Arabic numbers (1, 2, 3...) indicate significant effects of salinity, and letters indicate significant differences among individual means. Due to mortality, no data is given for 51 or 60g/l at 15°C. Error bars indicate standard error of the mean (n = 7).
Figure 4.2. The effect of simultaneous salinity (35, 43, 51, and 60 g/l) and temperature (15, 25, and 35°C) transfer on muscle water content in tilapia hybrids after 24 h. Due to mortality, no data are given for 51 or 60 g/l at 15°C. See legend of figure 4.1 for further details.
The effect of two week temperature acclimation on branchial Na\(^+\), K\(^+\)-ATPase in seawater (35 g/l)-exposed tilapia.

Branchial Na\(^+\), K\(^+\)-ATPase activity was affected by assay temperature in vitro, and there were significant interaction effects between acclimation and assay temperature (p < 0.001) (Fig. 4.5). In fish acclimated to 25 and 35°C for two weeks, Na\(^+\), K\(^+\)-ATPase activity was reduced by 86 and 89% respectively when assayed at 15°C. When assayed at 25°C, the 25°C-acclimated gills showed the highest rate of activity, while at 35°C, the 35°C-acclimated gills showed the highest rate of activity. 25°C-acclimated gills did not show an increase in activity when assayed at 35°C (Fig. 4.3).

Discussion

Effect of Combined Temperature and Salinity Challenges

The objective of this study was to assess the acute effects of a 24 h hypersalinity challenge at various temperatures. At 25°C tilapia plasma osmolality was maintained 24 h after transfer to all salinities (Fig. 4.1). Mozambique tilapia have previously been observed to regulate plasma osmolality within 24 h following transfer from freshwater to 20 g/l and from 20 to 30 g/l salinity at constant temperature (Hwang et al. 1989). In the current study, tilapia hybrids were unable to maintain plasma osmolality after exposure to elevated salinity at 35°C, and had a high rate of mortality at salinities greater than 47g/l at 15°C. Rapid transfer to elevated environmental salinity in teleosts commonly results in an increased plasma osmolality due to osmotic water loss.
Figure 4.3. In vitro branchial Na\(^+\), K\(^-\)-ATPase activity assayed at 15, 25, and 35°C in tissues isolated from tilapia hybrids acclimated to 15, 25, or 35°C for two weeks prior. Roman numerals (I, II, III) indicate significant effects of assay temperature, Arabic numbers (1, 2, 3...) indicate significant effects of acclimation temperature, and letters indicate significant differences among individual means. Error bars indicate standard error of the mean (n = 7).
and diffusive ion gains (Assem and Hanke 1979, Hwang 1987, Hwang et al. 1989, Kültz and Jurss 1993, Dang et al. 2000, Daborn et al. 2001), and euryhaline species generally respond with proliferation and/or hypertrophy of branchial MRCs, which is the primary site of NaCl excretion (Foskett et al. 1981, Perry 1997, Marshall and Bryson 1998). In acute transfer such as these, however, the contribution of gill morphological changes was most likely minimal. Interestingly, there were no significant changes in muscle water content, although plasma osmolality was significantly increased, which was also observed previously (see chapters 2 and 3).

Mechanisms of Physiological Impairment

High mortality of tilapia hybrids exposed to salinities above 47g/l at 15°C was most likely due to osmoregulatory stress (Al Amoudi et al. 1996). Low temperatures have been shown to result in depressed activity of branchial Na\(^+\), K\(^+\)-ATPase (Handeland et al. 2000), which has been shown to play a role in acute salinity challenges by rapid activation as soon as after 3 h of exposure in euryhaline fishes (Hwang et al. 1989, Mancera and McCormick 2000).

Osmoregulatory disturbances at 15 and 35°C may have also resulted from changes in membrane integrity. Acclimation temperatures above optimal for rainbow trout (Oncorhynchus mykiss) and Nile tilapia (Oreochromis niloticus) have been shown to result in voids within the membranes following alterations in lipid structure, and increased water and ion permeability have been attributed to these voids (Johnston and Cheverie 1985). Phase changes within the membrane at both extremes can lead to breaches that allow water and solutes to move through, and, in addition, may alter lipid
domains associated with normal enzyme function (Handeland et al. 2000, Hochachka and Somero 2002).

There were significant increases in plasma osmolality and \([\text{Na}^+]\) at 35°C. Teleosts subjected to an ambient temperature increase typically experience an elevated oxygen demand due to increased metabolic rate, and as a result, cardiac output increases to meet the demand (Barron et al. 1987). Increases in metabolism, usually associated with hypoxia or exercise, result in a recruitment of unperfused gill lamellae surface area, which increases overall ion diffusion (Holeton and Randall 1967, Hayton and Barron 1990, Handeland et al. 2000). Increased branchial ventilation rate decreases the stagnant water layer around the gill epithelium, and reduces a major physical barrier to ion diffusion (Hayton and Barron 1990). This osmoregulatory-respiratory compromise has been widely investigated in freshwater adapted fish; however, fish adapted to saline water also experience difficulties under these conditions (Kieffer and Tufts 1996, Handeland et al. 2000). Interestingly, at 35°C the changes in plasma \([\text{Na}^+]\) were much greater than those observed for \([\text{Cl}^-]\). Fish transferred to more saline water experienced a metabolic acidosis, and recovery of pH is associated with an increased strong ion difference (Smatresk and Cameron 1982, Wilkes and McMahon 1986, Maxime et al. 1991), which may be accomplished by a relative increase in \([\text{Na}^+]\), decrease in \([\text{Cl}^-]\), or a combination of the two. The relatively minor effect of salinity transfer on plasma \([\text{Cl}^-]\) and the large effect on \([\text{Na}^+]\) were likely indicative of blood pH compensation associated with hypersaline exposure.

Acclimation of fish to 15°C for 2 weeks did not alter \textit{in vitro} \(\text{Na}^+\), \(\text{K}^+\)-ATPase activity, relative to fish acclimated at higher temperatures, when all three were assayed at
15°C. Handeland et al. (2000) attributed a lowered capacity for ion transport, observed in Atlantic salmon (*Salmo salar*) exposed to 4.1°C, in part, to a reduced Na\(^+\), K\(^+\)-ATPase activity in response to lowered enzyme activity. In fish acclimated to 35°C, gill Na\(^+\), K\(^+\)-ATPase activity measured at 35°C was significantly elevated relative to fish acclimated at 15 and 25°C, indicating that there was an ability to upregulate enzyme activity, at least to the levels observed at 35°C. The large increase in activity observed between 35°C-acclimated gills assayed at 25 and 35°C, and the lack of similar increase in activity from 15 and 25°C-acclimated gills, may indicate the presence of more than one Na\(^+\), K\(^+\)-ATPase isoform. While previous studies have shown that different isoforms of this enzyme are expressed in FW- versus SW-acclimated fish (Lee et al. 1998), based on the results in the current study, isoform switching between fish exposed to different acclimation temperatures may have also occurred, and is worthy of further investigation.

The results obtained in experimental series I may be partially explained by the results of series II. When gills from 25°C-acclimated fish were assayed at 25 and 35°C, there was no change in activity associated with temperature, just as no change in plasma osmolality was observed when fish from 35 g/l at 25°C were transferred to 35 g/l at 35°C. However, when assayed at 15°C, these gills showed a near 86% decrease in activity. This drop in enzyme activity may be in part responsible for the large increase in plasma osmolality observed when fish acclimated to 35 g/l salinity at 25°C were transferred to 15°C.
Summary

Based upon mortality and sub-lethal indicators of osmoregulatory stress during transfer to increased salinity, this study clearly demonstrated that temperature has a substantial influence on salinity tolerance in both seawater-acclimated tilapia hybrids and those transferred to a hypersaline environment. While these fish were capable of surviving direct transfer from 35 to 60 g/l at 25 and 35°C, extensive mortality was observed when fish were transferred from 35 to 51 or 60 g/l at 15°C. Furthermore, direct transfer from 35 g/l to all salinities resulted in significant elevations in plasma osmolality [Na+] at 35°C relative to 25°C. Results of 24 h challenges indicated that osmoregulatory ability may have been compromised when tilapia hybrids were exposed to either extreme of their temperature range. The current study may be limited, however, as it investigated effects up to 24 h post-transfer, and an experiment involving longer exposure durations may better reveal temperature limitations on recovery following transfers to increased salinity.
Chapter Summary

1) A ±10°C change had a clear effect on osmoregulatory ability of this species. The transition point that was identified in Chapters 2 and 3 was much lower when transfers are not carried out at 25°C
   a. Transfer to 51 and 60 g/l at 15°C resulted in mortality, in spite of previous results showing that tilapia are capable of handling these salinity transfers at 25°C and 35°C. Transfer to 35 and 43 g/l resulted in higher plasma osmolality and ion concentrations.
   b. 35°C did not result in mortality, but tilapia were not able to maintain plasma osmolality at the same levels as those transferred to elevated salinity at 25°C

2) Muscle water content did not decrease significantly regardless of the magnitude of the salinity challenge or temperature, which has now been observed in both acute and gradual transfers.

3) There was a differential pattern of NKA activity between fish acclimated to 15, 25, or 35°C, which was evident by assaying at the various temperatures.
References


CHAPTER 5: TEMPERATURE CHANGE RESULTS IN OSMOTIC IMBALANCE IN THE CALIFORNIA MOZAMBIQUE TILAPIA
(Oreochromis mossambicus x O. urolepis hornorum): A PHYSIOLOGICAL BASIS FOR WIDE-SCALE MORTALITY IN THE SALTON SEA?

Introduction

The Salton Sea is a large (980 km²) inland lake that formed in the early twentieth century when the Colorado River flooded the Imperial Valley, forming a large inland sea in an arid desert. Due to inflow of nutrient and salt-rich water, and a very high rate of evaporation, the salinity of the Salton Sea has increased from near freshwater at the time of its formation to 44 g/l presently, and continues to increase at a rate of 0.3 g/l/year (Watts et al., 2001). Despite the physiological challenges of living in a hypersaline system, there has been a substantial fish population in the Salton Sea for decades. Over thirty species have been introduced into the sea over the last 80 years, however, it has been the orange mouth corvina (Cynoscion xanthulus), bairdiella (Bairdiella icista), and sargo (Anisotremus davidsoni) that have flourished since the mid-1950s (Walker et al. 1961). The dominant fish species over the last forty years has been a Mozambique tilapia hybrid (Oreochromis mossambicus x O. urolepis hornorum), which has been referred to as the California Mozambique tilapia (Costa-Pierce and Doyle, 1997).

These four species comprise a large portion of the Salton Sea fishery, and have all been shown to tolerate salinities greater than seawater (SW) (Hanson, 1970; Sardella et al., 2004b). However the steadily rising salinity of the sea has been implicated as a probable cause of the large scale fish kills that have plagued the Salton Sea fishery since 4

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the early 1990's (Kaiser, 1999). Fish kills have been observed in both summer and
winter months, with summer kills typically composed of all four species, and winter kills
composed primarily of tilapia (Hurlbert, unpublished MSS). While the salinity tolerance
of these species, as assessed by laboratory studies (Hanson, 1970; Sardella et al., 2004b),
was slightly higher than the current salinity of the Salton Sea, there are many additional
environmental stressors in the Salton Sea that can have additive effects with the high
osmotic gradient. Other stressors playing synergistic roles with high salinity in the Salton
Sea include eutrophication, a large seasonal temperature range (12-35°C), high [H₂S] (up
to 5 mg/l), high [NH₃] (1.2 mg/l), toxic metals such as selenium and arsenic, low
dissolved oxygen levels with occasional anoxia, and various disease outbreaks (Gonzalez
et al., 1998; Riedel et al., 2002; Watts et al., 2001).

Mozambique tilapia are well known for high salinity tolerance, as well as low
levels of dissolved oxygen (Sardella et al., 2004b; Stickney, 1986), however, their low
tolerance of cold ambient temperatures, such as those observed in the Salton Sea
throughout the winter, have also been documented (Al Amoudi et al., 1996; Sardella et
al., 2004a). We hypothesize that the low ambient temperatures in the Salton Sea during
the winter is a large contributing factor to fish kills due to the effect changes in
temperature have on osmoregulatory mechanisms. The Salton Sea has a wide annual
temperature range (12-35°C), and because there is essentially no thermal stratification,
fish are unlikely to avoid temperature extremes (Watts et al., 2001). At 25°C, California
Mozambique tilapia were able to tolerate salinities as high as 95 g/l, with sub-lethal
disturbances observed in salinities greater than 65 g/l (Sardella et al., 2004b). In the few
studies conducted to date, temperature has been shown to have a large effect on salinity
Mozambique tilapia are considered eurythermal, but their survival and osmoregulatory ability are reduced when environmental water temperature is near their lower limit (Al Amoudi et al., 1996; Allanson and Bok, 1971; Sardella et al., 2004a). Changes in temperature can result in instantaneous and dramatic changes in metabolism, membrane fluidity and integrity, and enzyme function; all of which can affect osmoregulation negatively (Al Amoudi et al., 1996; Almansa et al., 2003; Robertson and Hazel, 1999).

The purpose of this study was to investigate the effect of temperature change on the osmoregulatory ability of 'California' Mozambique tilapia, in order to investigate whether temperature-induced osmoregulatory disturbances may be a contributing factor to winter die-offs. Fish were acclimated to 43 g/l (the current Salton Sea salinity) before transfer to 15 or 35°C for 5 d. Plasma osmolality, [Na+] and [Cl−], muscle water content, and branchial Na+, K+-ATPase (NKA) were measured at 0, 24, and 120 h following transfer. To demonstrate how temperature stress can negatively affect what is generally a mild salinity challenge, a second transfer was conducted where tilapia were exposed to a combined temperature and salinity transfer; fish were acclimated SW (33 g/l) at 25°C and then transferred to 43 g/l at 15, 25, or 35°C. Plasma osmolality and branchial NKA activity were measured in these fish at 0, 3, 6, 12, and 24 h following transfer.

Materials and Methods

Experimental Animals

California Mozambique tilapia were donated by Pacific Aquafarms, in Niland, CA, and transported to San Diego State University, in San Diego, CA, where all
experiments were conducted. Fish were freshwater reared (~5 g/l) and then acclimated to 35 g/l (SW) gradually over 3 weeks according to Sardella et al. (2004b). All tanks were filtered by mechanical, chemical, and biological systems, aerated using submersible air stones, and held at a constant temperature of 25°C; salinity was manipulated using Instant Ocean synthetic sea salts.

Experiment 1: The effect of Temperature Transfer (±10°C) on 43 g/l-acclimated Tilapia

Thirty-five SW-acclimated tilapia were transferred to 43 g/l at 25°C and allowed to acclimatize for two weeks. Following acclimation, tilapia were transferred from 25°C to 15 or 35°C at 43 g/l salinity. Seven tilapia were sampled prior to transfer, and seven fish were sampled at each temperature 24 and 120 h following transfer.

Experiment 2: The Effect of Temperature Transfer (±10°C) during Simultaneous Salinity Transfer (SW to 43 g/l)

After 3 weeks of acclimation to SW at 25°C, seven fish were removed and terminally sampled. Twenty-eight SW-acclimated fish were transferred from SW at 25°C to 43 g/l at 25°C, with seven fish being removed and sampled at 3, 6, 12, and 24 h following transfer. This transfer protocol was then repeated with transfers from SW at 25°C to 43 g/l at either 15 or 35°C.

Sampling

Fish were killed by a lethal dose of benzocaine anesthetic, previously dissolved in 70% ethanol, and then diluted to a final concentration of 0.7 g/l. The caudal peduncle was severed, and blood was collected from the caudal vein into heparinized
microhematocrit centrifuge tubes, which were subsequently centrifuged in a Damon IEC MB microhematocrit centrifuge, and plasma was expelled into Microcentrigue tubes. In experiment 1 plasma osmolality was measured using a Wescor 5500 vapor pressure osmometer (Wescor Inc. Logan, Utah, USA), plasma [Cl\(^{-}\)] was measured using the colorimetric mercuric thiocyanate method (Zall et al. 1956), and plasma [Na\(^{+}\)] was measured with an atomic absorption spectrophotometer (Perkin Elmer model 3100 A). Muscle water content was determined by removal of approximately 1 g of posterior epaxial muscle, which was rinsed and patted dry, then placed into a pre-weighed scintillation vial and dried in a 70°C oven. The difference in weight was divided by the original wet weight and \times 100\%. Lastly, the second and third left gill arches were collected, frozen quickly on dry ice, and stored at -80°C for later analysis of NKA activity. In experiment 2, plasma was used only for measurements of plasma osmolality.

Na\(^{+}\), K\(^{+}\)-ATPase activity

Gills were homogenized in ~1 ml of SEID buffer (250 mM sucrose, 10 mM EDTA-Na\(_2\), 50 mM imidazole, pH 7.3, 0.05% deoxycholic acid). Branchial NKA activity was determined using a modified McCormick (1993) method and expressed as \(\mu\)mol of ADP per hour per \(\mu\)g total protein. Protein was measured using the Biuret method.

Statistical Analyses

A two-way analysis of variance (ANOVA) was used to determine the effects of temperature and time, or temperature and salinity of exposure on all parameters. Statistical tests were performed using SigmaStat version 3.0, with an \(\alpha\) value of 0.05
A Holm-Sidak post-hoc test was performed following significant two-way ANOVA results.

Results

Experiment 1: The effect of Temperature Transfer (±10°C) on 43 g/l-acclimated Tilapia

In 43 g/l salinity-acclimated fish, transfer to 15 or 35°C did not result in loss of orientation or mortality. There was a significant interaction of time and temperature on both plasma osmolality (F=9.31, p<0.001, d.f.=2) and [Na$^+$] (F=9.21, p<0.001, d.f.=2), however plasma [Cl$^-$] did not change significantly (Fig. 5.1). Both osmolality and [Na$^+$] peaked at 24 h in 15°C water, and osmolality recovered to near pre-transfer values 120 h following transfer (Fig. 5.1a), while plasma [Na$^+$] decreased from the peak value to a level still significantly greater than pre-transfer values (Fig. 5.1b). Plasma osmolality in 35°C exposed fish did not change in a similar fashion to 15°C exposed fish, and was not significantly elevated relative to pre-transfer values until 120 h following transfer. However, plasma [Na$^+$] in 35°C exposed fish was significantly increased at 24 and 120 h following transfer, but not to the same levels as those seen in 15°C exposed fish. Again plasma [Cl$^-$] showed no significant changes with temperature transfer (Fig. 5.1c). Muscle water content was significantly affected by time of exposure after fish were exposed to 15 and 35°C (F=6.13, p=0.005, d.f.=2). At 24 h, muscle water content was temporarily reduced relative to the values measured at 0 and 120 h (Fig. 5.2). Lastly, there were no changes in branchial NKA activity at 24 or 120 h following temperature transfer (Pooled Value = 7.69±0.62 μmol/h/μg protein).
Figure 5.1. The effect of a ±10°C temperature change on A) plasma osmolality, B) Plasma [Na⁺], and C) plasma [Cl⁻] in California Mozambique tilapia acclimated to 43 g/l salinity. Letters indicate significant differences between fish at 15 compared to 35°C at a given time, while numbers denote significant differences relative to time zero within a given temperature as determined by two-way ANOVA (p<0.001, n=7).
Figure 5.2. The effect of a ±10°C temperature change on muscle water content in California Mozambique tilapia acclimated to 43 g/l salinity. There was a significant effect of time as determined by two-way ANOVA* (p<0.01, n=7).
Experiment 2: The Effect of Temperature Transfer (±10°C) during Simultaneous Salinity Transfer (SW to 43 g/l).

There was no mortality or loss of equilibrium in tilapia transferred from SW at 25°C to 43 g/l salinity at 25 or 35°C. However, when fish were transferred to 15°C, there was 100% mortality prior to the 24 h sampling time. Plasma osmolality was significantly affected by time/salinity (F=119.281, p<0.001, d.f.=4), temperature (F= 24.53, p<0.001, d.f.=2), and time-temperature interaction (F=94.99, p<0.001, d.f.=8; Fig. 5.3). In fish transferred to 15°C, plasma osmolality increased at each time point up to 12 h, while fish at 35°C showed a significant increase in plasma osmolality by 6 h after transfer, which remained elevated over 24 h. In contrast, fish transferred to 25°C maintained plasma osmolality over the entire time course.

Branchial NKA was significantly affected by time/salinity (F=5.96, p<0.001, d.f.=4), temperature (F=17.55, p<0.001, d.f.=2), and time-temperature interaction (F=7.71, p<0.001, d.f.=8) (Fig. 5.4). Tilapia transferred at 25°C had higher NKA activity values at 3 h, which remained elevated at 12 h before beginning to decline by 24 h. The fish transferred at 15 and 35°C also showed an increase in NKA activity at 3 h, but it was decreased to pre-transfer levels by 6 h. While NKA from 15°C-acclimated tilapia remained low up until the death of the fish, the 35°C-acclimated fish showed a second increase in NKA activity, which was significantly higher than pre-transfer values by 24 h.
Figure 5.3. The effect of transfer from 35 to 43 g/l salinity at 15, 25, and 35°C on plasma osmolality in California Mozambique tilapia. Letters indicate significant differences due to temperature at a given time, while numbers denote significant differences relative to time zero within a given temperature as determined by two-way ANOVA (p<0.001).
Figure 5.4. The effect of transfer from 35 to 43 g/l salinity at 15, 25, and 35°C on branchial NKA activity in California Mozambique tilapia; all assays were carried out at 25°C. Letters indicate significant differences due to temperature at a given time, while numbers denote significant differences relative to time zero within a given temperature as determined by two-way ANOVA (p<0.01).
Discussion

Since 1992, semi-annual fish kills have been observed at the Salton Sea (Kaiser, 1999). While summer die-offs have involved multiple species, winter kills have primarily involved the tilapia hybrid. Based on the results from this and previous studies, the effect of reduced water temperature on osmoregulation during winter months is potentially a large contributor to such high seasonal mortality. Our results agree with previously published work showing that the effects of the interaction of temperature and salinity are a greater osmoregulatory stress than either stress alone (Al Amoudi et al., 1996; Allanson and Bok, 1971; Sardella et al., 2004a).

Experiment 1 showed the effects of a temperature transfer at constant salinity, in this case the salinity of the Salton Sea\(^5\). Plasma osmolality and \([\text{Na}^+]\) following temperature change increased in a similar fashion to what is typically observed following salinity increase (Fig. 5.1). The increase in plasma osmolality and \([\text{Na}^+]\) was associated with a decrease in muscle water content, as the high osmotic pressure draws water out of the intracellular compartment. Observation of muscle water loss in this species does not occur following a salinity increase at 25°C, even to salinities as high as 95 g/l (Sardella et al., 2004b). Temperature has been shown to have detrimental effects on osmoregulatory ability in that it can alter enzyme kinetics, disrupt membrane dynamics, and alter the diffusion gradient across the gill due to compromises between osmoregulation and respiration confounded by changes in metabolic rate (Hochachka and Somero, 2002; Gonzalez and McDonald, 1992; Randall et al., 1972). 120 h following temperature

\(^5\) We used Instant Ocean Synthetic Sea Salt to manipulate salinities in this experiment. It should be noted that the ionic composition of the Salton Sea differs from Instant Ocean at common salinity; Salton Sea water typically contains approximately two-fold calcium ion and three-fold sulphate ion. In previous experiments (see Appendix I), I have shown the effects of these differences are negligible.
transfer there was no difference in plasma osmolality between fish transferred to 15 versus 35°C, but the temporary disturbance at 24 h was greater in 15°C exposed fish, indicating that a decrease in temperature was a greater challenge relative to a comparable increase in temperature. This was observed in temperature transfer at constant salinity and the combined salinity/temperature transfer, where mortality was observed at 15°C. These data were consistent with previous studies with this species (Al Amoudi et al., 1996; Allanson and Bok, 1971; Sardella et al., 2004a).

Although an abrupt temperature change represents a worse-case scenario with respect to its effects on osmoregulation, we exposed tilapia to a combined temperature/salinity challenge for 24 h based on previous findings that the first 12 h are the most crucial for tilapia acclimation (Hwang et al., 1989; Hwang, 1987). In this experiment, tilapia were able to maintain osmotic balance following a 10 g/l increase in salinity at constant temperature for up to 24 h (Fig. 5.3), with minimal, yet significant increases in branchial NKA activity at 12 h (Fig 5.4), which has been previously described as the end of the ‘crisis period’ for this species (Hwang et al., 1989). Tilapia did not maintain plasma osmolality at the same level when temperature was increased to 35°C; plasma osmolality increased by 3 h following transfer, peaked at 12 h, and was still significantly elevated by 24 h (Fig. 5.3). Again, the peak in osmolality coincided with what Hwang et al. (1989) described as the crisis period with regard to salinity acclimation. The increased osmolality in fish held at 35 ºC relative to 25ºC values is likely associated with the temperature-induced increase in metabolic rate (Q_{10} effects). Under these conditions, gill water and blood flow were elevated to meet the demand of metabolism, which subsequently increased the potential for osmotic equilibration across
the gill epithelium (Gonzalez and McDonald, 1992; Randall et al., 1972). The enhanced osmotic gradient resulting from hypermetabolism offsets the advantage of increased gill NKA activity due to temperature (Hochachka and Somero, 2002; Sardella et al., 2004a).

Fish transferred at 15°C did not survive past the crisis period, with 100% mortality after 12 h of exposure. Prior to death, fish from this treatment group had steadily increasing plasma osmolality (Fig. 5.3). The likely cause of the mortality was loss of osmoregulatory control (Al Amoudi et al., 1996; Allanson and Bok, 1971), as NKA activity is essentially zero when assayed at 15°C (Sardella et al., 2004a). These data provide a clear demonstration of how an additionally imposed stress can complicate what has been previously observed to be a simple salinity acclimation.

A rapid activation of gill NKA has been observed in this species (Hwang et al., 1989; Sunny and Ommen, 2001) as well as the common killifish (Fundulus heteroclitus) (Mancera and McCormick, 2000; Towle et al., 1977), which is also a well known model of euryhalinity. Tilapia in all temperatures showed an initial increase in NKA at 3 h that is consistent with this prediction, but the fish with the additional imposed temperature stress were unable to maintain the elevated activity (Fig 5.4.), which may have ultimately led to elevated plasma osmolality. The cause behind the inability to sustain NKA activity is unclear.

The results clearly show that an abrupt temperature change can have a profound effect on osmoregulatory ability in this tilapia species, and that a combined temperature/salinity challenge was more stressful to osmoregulation than when either stress was applied alone. Tilapia exposed to 15°C in this study as well as in previous studies have been shown to experience osmoregulatory difficulty, and temperature within
the Salton Sea during the winter months has been observed to decrease in some years even below this value (Watts et al., 2001). While temperature changes in the Salton Sea are more gradual relative to laboratory experiments, these data provide a good example of how temperature shifts can negatively affect osmoregulation. I have only investigated two of the multiple stressors that exist in the Salton Sea. Additional simultaneous stressors may have further additive effects, for example in a previous study, tilapia were acclimated to water collected from the Salton Sea at 25°C, and when the temperature was reduced to 15°C, 100% mortality by 3 h resulted (see Appendix I).

The use of brackish areas of the Salton Sea by tilapia during periods of low temperature could possibly an attempt to alleviate temperature-induced osmoregulatory disturbances, but direct evidence to confirm this has yet to be collected. Further investigation into the possible selection of lower salinities when faced with a temperature stress would prove interesting. Based on the experimental findings of this and other studies involving abrupt temperature challenges, it is not unreasonable to conclude that the decreased temperature during the winter months is a major contributor to mortality events in the Salton Sea.
Chapter Summary

1) A ±10°C temperature change at constant salinity has similar effects on plasma osmolality as an increase in ambient salinity; plasma osmolality is increased directly following temperature transfer and recovers after 5 d.

2) Although it was shown previously that tilapia acclimate quickly following a 35 to 43 g/l magnitude salinity challenge at constant temperature of 25° C, when this transfer is conducted at 15 or 35°C there are large osmoregulatory disturbances.

3) Based on data obtained during 15°C transfers, it appears that low ambient temperatures may play a major role in tilapia kills that occur during the winter months at the Salton Sea.
References


CHAPTER 6: THE USE OF TISSUE MICROARRAYS TO ASSESS THE MITOCHONDRIAL-RICH CELL CHARACTERISTICS OF MOZAMBIQUE TILAPIA (*Oreochromis mossambicus*) FOLLOWING TEMPERATURE AND SALINITY STRESS.\(^6\)

Introduction

Exposure to temperatures outside of an organism’s optimal thermal range can have deleterious effects, which is often manifested in part as loss of osmotic balance in fish (Al Amoudi et al., 1996). The euryhaline cichlid Mozambique tilapia (*Oreochromis mossambicus*), while renowned as one of the most saline-tolerant teleost species in the world (Stickney, 1986; Sardella 2004a), has previously been shown to experience osmotic disturbances when exposed to temperature extremes in both fresh (Al Amoudi et al., 1996) and saline waters (Sardella et al., 2004b). Although studies investigating the effects of temperature on osmoregulatory ability are few, osmotic stress resulting from temperatures outside of the thermal tolerance range have been documented in juvenile turbot (*Scophthalmus maximus*; Imsland et al., 2003), common carp (*Cyprinus carpio*; Metz et al., 2003) and Atlantic salmon (*Salmo salar*; Staurnes et al., 2001). Mechanisms of temperature-induced osmoregulatory disturbances have been attributed to altered enzyme kinetics, altered phospholipid states resulting in a loss of membrane integrity, (Handeland et al., 2000; Hochachka and Somero, 2002; Johnston and Cheverie, 1985), and compromises between osmoregulation and respiration at the gills (Sardella and Brauner, in press; Gonzalez and McDonald 1992, 1994, Randall et al., 1972). Osmotic stresses that result from temperature changes must be dealt with in similar fashion to the response of fishes following salinity changes. In FW teleosts, this typically involves the

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\(^6\) A version of this chapter has been submitted to the Journal of Experimental Biology.
cessation of drinking and increased branchial Na\textsuperscript{+}, K\textsuperscript{+}-ATPase (NKA) levels within mitochondrial-rich cells (MRCs). In SW, drinking rate typically increases to offset the loss of water, and SW MRCs expend metabolic energy to excrete ions.

The purpose of this study was to investigate the effects of temperature acclimation on the characteristics of MRCs and enzyme activity in seawater (SW)- and freshwater (FW)-acclimated Mozambique tilapia. We used tissue microarrays (TMA) to measure NKA content of gill MRCs, as well the NKA content within the renal tubule, and laser-scanning cytometry (LSC) of live MRCs to measure number and size following acclimation. This was the first application of these novel techniques to investigate the osmoregulatory responses to disturbance resulting from temperature in a euryhaline fish species. We measured plasma osmolality, as well as branchial and intestinal NKA activity in fish acclimated to 15, 25, and 35\textdegree C. NKA was assayed at various temperatures in order to determine how temperature affects osmoregulatory status and NKA kinetics.

TMAs allowed us to address the hypothesis that SW fish have a higher NKA content per MRC with direct measurements. Previous studies with tilapia have shown this indirectly; seawater-acclimated tilapia have a higher NKA activity following a transfer from FW to SW (Hwang et al., 1989), while Cioni et al. (1991) showed no significant difference in MRC number between FW and SW. Using the TMA technique, we were able to make a direct comparison of the NKA content of MRCs between FW and SW-acclimated animals, as well as assess the effects of exposure to sub-optimal temperatures.
Materials and Methods

Fish

Mozambique tilapia (*Oreochromis mossambicus*) were reared in non-chlorinated, University of California, Davis, well water at the Center for Aquatic Biology and Aquaculture (CABA), at 28°C, and weighed 9.80±1.1 g. Prior to experiments, approximately 50 tilapia were seawater-acclimated (SW) in two steps; fish were transferred from 0 to 15 g/l salinity for four days, and then from 15 to 33 g/l, and allowed to acclimate for 14 d. Salinity was manipulated using Instant Ocean synthetic sea salt, and no mortality was observed during acclimation.

Tilapia were acclimated to FW or SW at 15, 25, or 35°C, yielding six treatments (n=8 fish per treatment) for 14 days. Fish were fed approximately 1% body weight per day, but food was withheld 24 h prior to sampling. All six treatments were duplicated to provide a complete set of gills for LSC counts. During temperature acclimation, two FW fish being held at 15°C died, resulting in n=6, but all other groups had n=8. After the 14 d exposure to temperature, fish were killed by a lethal dose of MS-222 (Sigma), and sampled.

Sampling

Blood was collected into microhematocrit capillary tubes and centrifuged in a Damon IEC MB microhematocrit centrifuge at 11,000 g for 3 min. Hematocrit was then recorded and tubes were cut so the plasma could be collected. Plasma osmolality was immediately measured using a Wescor 5520 vapor pressure osmometer (Wescor Inc. Logan, Utah, USA). Tilapia were dissected and the second and third right gill arches and
the duodenal intestine, with the contents removed, were frozen in liquid N\textsubscript{2} for use in NKA assays, while left gill arches and whole kidney were fixed in 4% paraformaldehyde for use in TMAs. Unfortunately, there was an insufficient amount of renal tissue to make both TMA and NKA activity measurements, therefore, only TMA data are available for renal tissue. In contrast, intestinal tissue is difficult to array due to its morphology, so only NKA activity data are available. Tilapia from the LSC groups were killed by a blow to the head, and all eight gill arches were removed. Blood was rinsed and/or wiped from the gill, and the epithelium was scraped off into Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free phosphate-buffered saline (PBS; 146 mM NaCl, 3 mM KCl, 15 mM NaHPO\textsubscript{4}, 15 mM Na\textsubscript{2}HPO\textsubscript{4}, 10 mM NaHCO\textsubscript{3}, pH=7.4).

\[ \text{Na}^+, \text{K}^+\text{-ATPase activity} \]

Branchial and intestinal tissues were homogenized in 1 ml of SEID buffer (250 mM sucrose, 10 mM EDTA-Na\textsubscript{2}, 50 mM imidazole, pH 7.3, 0.05% deoxycholic acid). Branchial and intestinal NKA activity was measured using a modified McCormick (1993) method and expressed as \( \mu \text{Mol of ADP per hour per } \mu \text{g total protein} \). NKA activity was measured at 15, 25, and 35°C in tissues from all acclimation temperature groups.

Tissue Microarray Construction and Immunohistochemistry

Immunohistochemical analysis using the TMA technique was used to measure the concentration of NKA in individual branchial MRCs and renal tissue sections. Using the method previously described by Lima and Kültz (2004), renal and branchial tissues were
dehydrated and fixed in paraffin using a Tissue Tek vacuum infiltration processor (Sakura Finetek, Torrance, CA, USA), and then embedded into paraffin blocks on a Tissue Tek tissue embedding center (Sakura Finetek, Torrance, CA, USA). Cores 1 mm in diameter were removed from an empty paraffin block using a MTA-1 tissue microarray (Beecher Instruments, Sun Prairie WI, USA), and filled with cores of tissue taken from donor blocks. Tissue cores from all treatment groups were placed within one gill or one kidney block. The microarray block was sectioned at 4μm thickness using a Bromma 2218 Histogenics microtome (LKB, Uppsala, Sweden), and sections were floated on to a poly-lysine coated glass microscope slide; slides were dried overnight at 44°C.

Once dry, slides were deparaffinized in xylene for 5 min (3X), 100% ethanol (2X), 95% ethanol (2X), and 80% ethanol (1X). Slides were then incubated in phosphate-buffered saline containing 1% BSA (blocking solution) for thirty minutes, followed by 60 min incubation in blocking solution containing α5, an anti-NKA purified mouse IgG. Primary antibody against avian Na⁺, K⁺-ATPase α-subunit developed by Douglas M. Fambrough was obtained from the Developmental Studies Hybridoma Bank instituted under the auspices of the National Institute for Child Health and Human Development (NICHHD) and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. The antibody was diluted to a concentration of 6.2 μg/μl in PBS with 1% bovine serum albumin, and slides were incubated for 60 min. Next the slides were incubated with a secondary goat anti-mouse antibody covalently bound to Pacific Blue (P-10993, Molecular Probes, Eugene, OR, USA) at a final concentration of 20 μg/ml. Slides were rinsed with PBS and
counterstained with propidium iodide following antibody incubations. Finally, cover slips were mounted on the slides using a ProLong Gold antifade reagent.

Live Cell Staining

Cell suspensions were stained with the mitochondrial stain dimethylaminostyrylmethylpyridiniumiodide (DASPMI; 10µM) for 30 minutes and washed. After resuspension in 100 µl of PBS, 80 µl was pipetted on to a microscope slide and covered with a 60x24 mm cover slip as described by Lima and Kültz (2004). MRC counts and measurement of area were then carried out on the LSC.

Laser Scanning Cytometry

A laser scanning cytometer (LSC, Compucyte, Cambridge, MA, USA) was used to quantify NKA abundance in individual branchial MRCs and renal tissue, as well as to calculate the mean area for individual branchial MRCs. Immunohistochemical analysis of TMAs was conducted using a 40x objective (UPlanFL 40x/0.75/∞/0.17, Olympus, Melville, NY, USA) in combination with the LSC UV laser (400 nm). Mitochondrial-rich cells were easily detected in the interlamellar regions of the gill due to high intensity of NKA/Pacific Blue fluorescence. Each fluorescing cell or cell fragment was considered an ‘event’, and contour lines were added around each event in order to obtain a measurement of size and fluorescent intensity of that event. Contouring and event detection variables were set for a minimal area of 50µm and maximum area of 200µm to avoid inclusion of cell fragments or multi-cellular complexes, respectively. Within the renal tubules, individual cells were difficult to contour by this method, therefore a
defined area of tissue called a “phantom” was randomly selected for quantification of NKA abundance. Phantoms are analogous to the contour lines drawn around the MRCs in the gill microarray analysis, so renal NKA abundance is also expressed per unit area. Determination of an average area and fluorescence was carried out using WinCyte software, and the intensity of NKA fluorescence is presented as relative fluorescent units (RFU).

LSC was also used to measure the number of MRCs per fish and the average size of MRCs by detection of the mitochondrial stain DASPMI. For live cell suspensions, we used a 20x objective (UPlanFL 20x/0.50/0/0.17, Olympus). Once again, contouring and event detection was optimized as described above, as well as in Lima and Kültz (2004).

Statistical Analysis

We used two-way analyses of variance (ANOVA) to analyze all of the variables tested. Significant two-way ANOVAs were followed by a Holm-Sidak post hoc test. All statistical analysis was carried out using Sigma Stat version 3.0, with $\alpha = 0.05$. Values are reported as mean±SE, n=8.

Results

Mortality was observed in fish acclimated to FW at 15° C; two of eight fish died during the two week acclimation period. Both FW and SW fish exposed to 15°C showed little spontaneous activity, and seldom ate the food presented to them. In the 25 and 35°C tanks, all fish were active, and consistently consumed the food they were provided. There was a small but significant difference in hematocrit with salinity (FW = 31.9±0.9%, SW = 35.2±1.2%); however, there was no effect of temperature.
Figure 6.1. Plasma osmolality in Mozambique tilapia following two weeks of exposure to 15, 25, or 35°C in SW (gray bars) and FW (white bars). Letters that differ indicate significant differences within an acclimation temperature and numbers that differ indicate significant differences within an acclimation salinity (n=8).
Figure 6.2. The effect of acclimation temperature on a) branchial and b) intestinal NKA activity in Mozambique tilapia. Assays were conducted at physiological temperature. * indicates a significant effect of salinity (SW>FW), and bar(s) indicate a significant effect of temperature (15°C<25°C<35°C in gill tissue, 15°C<25°C<35°C in intestinal tissue; n=8). Comparisons between individual specific means could not be conducted due to lack of interaction effects.
Plasma osmolality was significantly affected by salinity (p<0.001), temperature (p<0.001), and temperature-salinity interaction (p<0.001). Osmolality was significantly increased in SW tilapia at 15°C, and significantly reduced in FW tilapia at 15°C (Fig. 6.1). Values from the remaining treatment groups were not significantly different.

Branchial NKA activity was significantly higher in SW-acclimated fish when assayed at physiological temperature (p<0.001; Fig. 6.2a). In addition, there was a significant effect of temperature on NKA activity, with values measured at 15°C being significantly lower than those from 25 and 35°C fish. There was no significant interaction of temperature and salinity. Intestinal NKA activity was significantly affected by temperature (p<0.001) and salinity (P<0.001), but there were no interaction effects. NKA activity was higher in SW fish, and increased with temperature (Fig. 6.2b).

Mitochondrial-Rich Cell Characteristics

Mitochondrial-rich cells were easily distinguished from surrounding cells by high intensity of NKA/Pacific Blue fluorescence found in the interlamellar regions. Several hundred events were detected in each tissue section on the microarray, and average fluorescent integral and event area were subsequently plotted on a scatter plot (see Lima and Kültz (2004) for more detailed description). Events with an area less than 50 μm² were discounted as they most likely represented either cell fragments or immature mitochondrial-rich cell forms, while events greater than 200 μm² in size were discounted as they most likely represented multi-cellular complexes Lima and Kültz (2004). Events within these limits were counted in our analysis as individual MRCs, and we averaged
Figure 6.3. The effect of temperature on NKA content (Relative Fluorescence) of mitochondrial-rich cells in tilapia exposed to 15, 25, or 35°C in FW and SW, assessed with the α5 antibody using tissue microarray. There was a significant effect of salinity (SW>FW) as denoted by * (n=8).

Figure 6.4. The effect of temperature on the size (μm²) of mitochondrial-rich cells in tilapia exposed to 15, 25, or 35°C in FW and SW, assessed by LSC detection of the mitochondrial stain DASPMI. SW MRCs were significantly larger than FW MRCs as denoted by *, and 15°C-acclimated tilapia had significantly larger MRCs relative to 25 and 35°C as denoted by the bar (n=7).
111.06±12.1 events per fish for analysis of relative differences between treatments.

Salinity had a significant effect on the abundance of NKA, with SW-acclimated MRCs having more NKA per MRC (Fig. 6.3).

Dissociated MRCs stained with DASPMI were segmented as individual events and contoured along their perimeter as described by Lima and Kültz (2004) and as described above for TMA analysis. The number of MRCs was highly variable amongst the treatment groups and proved to be inconclusive (data not shown); however, there were significant effects of salinity and temperature on MRC size (p<0.001 and p=0.023, respectively; Fig. 6.4). SW MRCs were larger than those from FW tilapia with acclimation to 15°C resulting in even larger cells (Fig. 6.4).

Renal NKA was identified by intense NKA / Pacific Blue fluorescence along the serosal membrane of the renal tubule in SW-acclimated tilapia (Fig. 6.5). Random areas referred to as “phantoms” were assigned to the scans of renal tissue, within these phantoms, the WinCyte software measured the relative fluorescence of the defined area, similar in method to the measurement within contoured MRCs in the gill scans. There was a significant effect of temperature (p=0.04), and temperature-salinity interaction (p=0.02) on renal NKA content. SW fish acclimated to 15°C had a significantly greater content of NKA relative to other treatments (Fig. 6.5).

Enzyme Kinetics

Branchial NKA activity in tissue taken from FW-acclimated tilapia was significantly affected by acclimation (p<0.001) and assay temperature (p<0.001), as well as interaction effects (p<0.001). Activity increased with assay temperature in all
acclimation groups (Fig. 6.6a). Gill tissue from SW-acclimated tilapia was also significantly affected by acclimation and assay temperature, with interaction between the two variables (all p<0.001). Interestingly, 15°C-acclimated gill tissue had the highest activity at all assay temperatures relative to 25 and 35°C-acclimated gills (Fig. 6.6b). When assayed at 35°C, gill tissue from 15°C-acclimated fish has an NKA activity rate nearly double that of 25 and 35°C acclimated gills (Fig. 6.6b); this relationship was not observed in FW-acclimated fish.

In contrast to gill tissue, intestinal NKA activity increased with assay temperature. In FW-acclimated intestine, there was a significant effect of acclimation and assay temperature, as well as interaction between salinity and temperature (all p<0.001; Fig. 6.7a). In SW-acclimated intestine, there was no effect of acclimation temperature, but activity was significantly increased with increasing assay temperature (p<0.001; Fig 6.7b).
Figure 6.5. a) The effect of temperature on the renal NKA content per μm² in tilapia exposed to 15, 25, or 35°C in FW and SW, assessed using the α5 antibody on tissue microarray. Tilapia acclimated to 15°C in SW had a significantly greater NKA content relative to all other groups (p<0.001; n=8). b) FW kidney stained with α5 antibody from tilapia exposed to 15°C for two weeks. c) SW kidney stained with α5 antibody from tilapia exposed to 15°C for two weeks; tissues were illuminated with an ultraviolet laser at 400 nm.
Figure 6.6. The effect of assay temperature (15, 25, or 35°C) on branchial NKA activity from Mozambique tilapia acclimated to 15, 25, or 35°C in a) FW and b) SW. Letters that differ indicate significant differences within acclimation groups due to assay temperature, and numbers that differ indicate significant differences among acclimation groups for each assay temperature. See text for individual p values (n=8).
Figure 6.7. The effect of assay temperature (15, 25, or 35°C) on intestinal NKA activity from Mozambique tilapia acclimated to 15, 25, or 35°C in a) FW and b) SW. See figure 6.6 for details.
Discussion

We have shown that the NKA content within MRCs was significantly higher in SW tilapia relative to FW (Fig. 6.3). To our knowledge, this is the first time that a difference in the direct measurement of NKA content between SW and FW MRCs has been measured; however, Lima and Kültz (2004) did observe an increase in NKA content in 2.4 X SW relative to FW and SW after 4 weeks in Fundulus heteroclitus. Previously, it has been speculated that SW MRCs contain higher levels of NKA based on higher levels of activity (Hwang et al., 1989; Weng et al., 2002), increased NKA mRNA expression, and increased protein content (Feng et al., 2002) of whole gill homogenates. In addition to higher NKA concentration per cell, SW MRCs were generally larger than FW MRCs (Fig. 6.4) similar to the findings of Foskett et al. (1981); larger cells with greater NKA concentration is supported by higher levels of NKA activity in SW-acclimated gill homogenates (Fig 6.2).

In both FW and SW, tilapia were able to acclimate to 35°C without showing signs of osmotic stress after 2 weeks. In spite of the increased metabolism typically associated with a 10°C increase in temperature, fish acclimated to 35°C had similar plasma osmolality values to 25°C-acclimated fish (Fig. 6.1) without increasing branchial NKA activity or abundance (Fig. 6.2a and 6.3, respectively). Maintenance of plasma osmolality without any difference in branchial NKA activity or abundance at this temperature may indicate some form of NKA modification in the gill or tightening of the branchium to prevent water loss, but this remains to be investigated further. Acclimation to this temperature probably indicates that 35°C is within the thermal tolerance range for this species with respect to osmoregulatory balance under resting conditions, although
shorter-term exposures to this temperature does result in osmoregulatory disturbances at higher salinities (Sardella et al., 2004b).

In contrast, 15°C-acclimated fish showed an inability to maintain plasma osmolality at levels observed at 25 and 35°C (Fig. 6.1). The altered plasma osmolalities in 15°C-exposed fish was likely a result of substantially lowered enzyme kinetics (Handeland et al., 2000), as NKA activity from all acclimation groups was consistently low when assayed at 15°C (Fig 6.2). Decreases in membrane fluidity have also been observed in fish exposed to lower than optimal temperatures, and fluidity changes can result in voids and/or ruptures in cell membranes (Johnston and Cheverie, 1985; Robertson and Hazel, 1999) and lead to further osmotic imbalance. Lastly, the low level of feeding observed in 15°C tanks may also contribute to the poor ion absorption in FW fish; however this was not measured directly.

It was clear from both qualitative and quantitative observations that tilapia were more susceptible to 15°C exposure, in particular, fish acclimated to FW. Cold temperatures were a greater challenge to FW relative to SW tilapia based on greater mortality and loss of orientation; we speculate that the greater NKA abundance and MRC size in SW fish explain this observation. The SW 15°C-acclimated fish generally had higher gill NKA activity levels at each assay temperature, relative to fish acclimated to 25 and 35°C, and exhibit a dramatic increase in gill NKA activity when assayed at 25 and 35°C relative to 15°C. When assayed at 35°C, the gill NKA activity in SW fish acclimated to 15°C is nearly twice that of 25 and 35°C acclimated fish, indicating some form of enhanced NKA transport capacity in this acclimation group. TMA data indicate that there was no upregulation of total NKA content when SW fish were acclimated to
15°C, however, there was an increase in MRC size that may partially account for the increased NKA activity relative to 25 and 35°C-acclimated tilapia. A larger number of MRCs would also explain this, but no data is available to suggest this. With a substantial increase in NKA activity with assay temperature in the 15°C SW group, the additional possibility of post-translational modification should not be ruled out. A further explanation for the increased capacity for NKA activity in 15°C-acclimated SW fish may be the utilization of inactive NKA that has been observed previously in this species following salinity challenges (Hwang, 1989). The α5 antibody is specific for the α-subunit of NKA, and may have bound to subunits that were non-functional in 25 and 35°C-acclimated fish giving the appearance of similar NKA content amongst the three temperature groups. Using killifish, Mancera and McCormick (2000) showed that the rapid activation was osmolality-sensitive. In our study, SW fish exposed to 15°C showed higher plasma osmolality relative to the 25 and 35°C following 2 weeks of acclimation. A chronic level of elevated plasma osmolality may result in activation of NKA in SW-acclimated MRCs; however, the additional activation was still insufficient to achieve similar plasma osmolality levels as measured in 25°C fish. Rapid activation has been previously described as a SW-acclimated phenomenon, which may partially explain why NKA activity patterns of the FW gill at 15°C do not follow a similar trend when assayed at various temperatures. Further investigation into this mechanism would certainly prove interesting. Finally, in addition to pre-existing benefits of SW acclimation at the gill, the SW-acclimated kidney also showed signs of acclimation to the osmotic stress of cold temperature.
While there was no change in renal NKA content in FW-acclimated tilapia, there was a significant increase in NKA content in the SW-acclimated tissue when fish were acclimated to 15°C. Although the kidney is more commonly regarded as the site for divalent ion excretion, the majority of the ions excreted at the kidney are Na⁺ and Cl⁻, and SW-acclimated fish at 15°C have higher levels of NKA in the serosal membrane of the renal epithelium relative to fish acclimated to 25 or 35°C. It would appear from the combined data that the SW fish attempt to compensate for a loss of osmotic balance by increasing the ion-transporting capacity of both the branchial and renal epithelia. Interestingly, there was no evidence to suggest that FW fish have such a response, which may partially explain the mortality observed in the FW group at 15°C, and the proportionally greater loss of osmotic balance in FW relative to SW at 15°C (as evident by loss of orientation).

In general the intestinal NKA activity was greater in SW relative to FW, which is most likely a result of the greater role of this enzyme in SW acclimation. NKA provides the driving force for the absorption of salts across the intestinal wall whereby water can be absorbed passively by osmosis (Wilson et al., 1996). Furthermore, there is evidence that the osmotic gradient is further enhanced by the secretion of bicarbonate into the gut lumen and subsequent formation of Ca²⁺ and Mg²⁺ precipitates (Wilson and Grosell, 2003; Wilson et al., 2002). Unlike branchial NKA, the intestinal NKA followed a predictable pattern of increase with assay temperature, this may be indicative of differences between NKA isoforms in these two crucial epithelia, but this remains to be studied in greater detail.
In summary, we have provided the first direct evidence, using TMA analysis, that SW fish have more NKA per MRC relative to FW fish. The data also support previous studies that have shown the importance of the intestinal epithelium in SW acclimation. The results also suggest that a 10°C decrease is more detrimental to tilapia than a 10°C increase over the range of temperatures used in this study, in particular when fish are acclimated to FW. In addition to a greater NKA abundance, we have shown that SW-acclimated fish have larger MRCs, and that these cells increase further in size during the osmotic stress resulting from a 10°C decrease in temperature. SW tilapia responded to the temperature change to a greater degree than FW, with physiological changes evident in both the branchial and renal epithelium. This may explain the greater mortality and loss of equilibrium that was observed in FW, and also why 15°C is a greater challenge to FW tilapia.
Chapter Summary

1) TMAs show that MRCs from SW-acclimated tilapia have more NKA per cell relative to FW-acclimated tilapia. Furthermore, live cell suspensions stained with the mitochondrial stain DASPMI were larger in SW-acclimated fish, and gill tissue from SW acclimated fish had higher NKA activity.

2) Relative to tilapia acclimated to 25 and 35°C, in 15°C acclimated fish, plasma osmolality was significantly reduced in FW and elevated in SW indicating a negative affect of 15°C on osmoregulation in this species.

3) SW-acclimated tilapia showed a measurable response to the low T induced osmotic stress; there was a hypertrophy of branchial MRCs and an upregulation of renal NKA content. Furthermore, when assayed at common temperatures, 15°C-acclimated fish had a much greater branchial NKA activity relative to. Lack of similar responses by FW-acclimated fish may be a cause for the mortality that was observed in this treatment group.

4) NKA activity assayed at 15°C was very low in all acclimation groups, and assuming that this assay is indicative of in vivo activity in fish at 15°C osmoregulatory capacity at this temperature in the Salton Sea would be expected to be minimal.
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CHAPTER 7: GENERAL SUMMARY AND CONCLUSIONS

The Salton Sea has experienced wide scale semi-annual fish kills that have decimated its once robust fishery. Following what has been called the 'millennium die-off', greater than 90% of the tilapia fishery was thought to have been lost (Riedel et. al., 2002; Hurlbert, unpublished MSS). There are now reports five years later that the tilapia population has begun to recover (Jack Crayon, CA Dept. of Fish and Game, personal communication). Winter month fish kills are typically specific to tilapia hybrids, to find large numbers of other species dead at this time of year is said to be 'unheard of' (Hurlbert, unpublished MSS). While rising salinity is certainly a contributing factor to Salton Sea fish kills, research from my thesis and previously published literature involving tilapia, have shown that the interaction with other stressors (most importantly temperature) creates a much greater challenge.

Currently, the reduction of water inflow to the sea is a contentious issue in Southern California, as water demand in the surrounding areas continues to escalate. Inflow reduction will accelerate the increase of the Salton Sea's salinity and destroy the majority of estuarine habitat. The use of these habitats by the Salton Sea fishes should be considered by management agencies prior to water flow reductions if maintenance of a viable fishery is a potential goal. Aggregation of tilapia in estuarine areas of the Salton Sea during cold months has been observed (Riedel et al., 2002), and may indicate that current salinity of the Salton Sea (~44 g/l) is greater than the preferred salinity of this species when temperature is below 25°C. Based on responses of tilapia during 15°C exposures in laboratory experiments (e.g., mortality or loss of orientation), aggregation of fish in estuarine areas may be an attempt to lessen the osmotic gradient and assuage the
effects of a deleterious temperature-salinity interaction. Fish kills during the hot summer months involve multiple species. During these months, fish have also been observed taking refuge in estuarine areas where rivers enter the Sea. Under these circumstances migration to estuarine areas is thought to be a refuge against hypoxia and/or anoxia in the pelagic areas (Reidel et al. 2002).

Although the salinity tolerance of the California Mozambique tilapia is extremely high, limitations exist with respect to its ability to handle abrupt changes. Kültz and Onken (1993) transferred pure Mozambique tilapia from 10 to 60 g/l, and while these fish could acclimate and survive, but the same species was not able to handle a direct FW to SW transfer (Hwang et al., 1989; Hwang, 1987). The transition from a hyper- to hypoosmoregulatory strategy may place limitations on the magnitude of the salinity increase that can be tolerated. Hwang et al. (1989) suggested that dehydration tolerance limits to the magnitude of salinity challenge, and that a challenge greater than FW to 25 g/l results in a lethal level. Currently, the move between the irrigation ditches and very small rivers (approximately 2 g/l) leading to the Sea into the Salton Sea proper represents very sharp (~39 g/l) increase in salinity and requires the animal to switch between a hyper- and a hypoosmoregulator. In lab experiments, this has proven to be fatal to this species, as well as to red belly tilapia (Tilapia zillii), another resident of the area (unpublished data). Because of this limitation, new tilapia are unable to invade the Sea from the surrounding areas in appreciable numbers, which is likely a contributing factor in the slow recovery of the Salton Sea populations following the fish kills such as the millennium die-off. Given the variability of responses by tilapia in dealing with multiple simultaneously-applied stressors (which is certainly the case in the Salton Sea), the
slowly and consistently changing environment, and lack of gene flow into the Sea due to the ‘salinity wall’, it is possible that two distinct populations of this tilapia species in the Coachella/Imperial Basin exist. As the preceding work was done almost entirely on California Mozambique tilapia obtained from Pacific Aquafarms in nearby Niland CA (thought to be the original source for the population of tilapia that reside in the Salton Sea; Costa-Pierce and Doyle, 1997), a comparison of osmoregulatory ability, as well as the genetic variation between these fish acclimated to elevated salinities and wild fish would prove extremely interesting.

The Salton Sea has a long list of environmental variables that can potentially act as stressors to fish. While I have focused primarily on two that seem likely to have the greatest effect on the California Mozambique tilapia; others such as hypoxia/anoxia, selenium, arsenic, ammonia, and disease, as well as the interaction of these stressors need to be included into any model of salinity tolerance for the Salton Sea fishery.

**Mechanisms of Hypersaline Tolerance in the California Mozambique Tilapia**

One of my most interesting findings was that this tilapia hybrid appears to possess two distinct osmoregulatory responses to elevated salinity. A transition between the two responses occurred at approximately 60 g/l and was observed during both short (5 d; chapter 2) and longer-term (up to 28 d; Chapter 3) acclimation experiments. The first response was elicited from tilapia by salinities from 15 to approximately 60 g/l. Throughout this range, there was no change in drinking rate, MRC turnover, or NKA activity; however, whole animal oxygen consumption decreased with salinity (see below). Despite the lack of upregulation of osmoregulatory capacity beyond levels observed in SW, plasma osmolality, [Na⁺], [Cl⁻] and muscle water content were
maintained in spite of a very high osmotic gradient with the environment. The lack of increase in drinking rate to levels greater than SW-acclimated animals indicates that water loss was also not increasing as tilapia were transferred to salinities above SW. Examination of electron micrographs showed morphology of the branchial epithelium also remained consistent with what was observed in a SW-acclimated fish throughout the range of salinities from 15 to 60 g/l, and thus a thickening of the epithelium to reduce water loss can be ruled out.

The reduced oxygen uptake rate with progressive elevations in salinity was observed in two separate experiments. In Chapter 3 it was associated with a quantitatively similar decrease in the activity of total hepatic ATPase activity. There are two possible explanations for this finding; first, that the decrease in liver ATPase is in response to an unknown signal (e.g., endocrine), and the decrease in activity subsequently led to a lower metabolic rate. The second is that there was a reduction in gill blood and water flow which resulted in internal hypoxemia and the liver responded accordingly reducing liver ATPase activity. While my data can not support or refute either hypothesis, the latter is very intriguing and is discussed in more detail below.

Teleosts have been shown to redistribute blood flow within the gill as a response to environmental conditions such as hypoxia (Holeton and Randall, 1967; Sundin, 1996). Blood flow redistribution is typically manifested as shifts between the arterioarterial and arteriovenous circulations, recruitment of unperfused lamellae, or redistribution of blood within the lamellae into basal regions where exposure to the environment is minimal (Holeton and Randall, 1967; Olson, 1991; Sundin and Nilsson, 1996), and these mechanisms have been shown to be under neuronal control (Bergman et al. 1974).
is no previous evidence that fish respond to elevated salinity by altering blood flow patterns; however, such changes may play a large role in the high salinity tolerance of Mozambique tilapia. By reducing the functional surface area (FSA) of the gill, via the limited lamellar shunting capacity of this species (Vogel et al., 1973), or via redistribution of lamellar blood flow toward more basal areas, the osmotic gradient across the gill epithelium can be lessened. FSA reduction could account for the lack of increase in water loss to the environment at higher salinities, evident by the lack of increase in drinking rate. It may also be associated with a reduction in whole animal oxygen consumption rate, which was observed in two separate experiments.

Reducing FSA during hypersaline exposure would allow for the maintenance of osmotic balance without upregulation of osmoregulatory mechanisms, but due to the nature of the osmo-respiratory compromise, oxygen uptake would be affected negatively. When temperature is increased from 25 to 35°C, a higher metabolic rate is forced upon the fish; tilapia had a progressively increased plasma osmolality relative to what was observed during salinity challenges conducted at 25°C, indicating how a higher metabolic rate can affect osmotic forces across the gill. The transition point between the two response patterns may represent the point when the osmotic gradient is too great for reducing blood and water flow to the gill to sufficiently maintain osmotic balance. At this point, the loss of integrity of the plasma compartment resulted in the up regulation of the often observed suite of osmoregulatory factors including branchial NKA, as well as drinking rate, paracellular junction surface area, and MRC turnover rate. The transition point also correlated with the observation of tight junction interdigitations between the apical regions of mature MRCs and their corresponding accessory cells within
multicellular complexes. Observations of interdigitated junctions were made after 5 d of exposure to 55 g/l salinity; associations of this type increase the overall surface area of the paracellular pathway, which is crucial to efficient Na\(^+\) excretion (Marshall, 2002). The increase of junctional surface area was the first of multiple changes. As salinity continued to be increased, production of new MRCs and MRC apoptosis (increased MRC turnover) was also increased. At 75 g/l, tilapia responded with an increase in drinking rate by 100% relative to what was observed at 55 g/l, and NKA activity increased by 122% relative to values at 65 g/l. Tilapia also showed signs of osmoregulatory stress, which were even more prevalent when salinity was increased to 85 and 95 g/l. At the highest salinities, there was extensive cell death by apoptosis and necrosis, and some death was observed when fish were held at this salinity for 14 d, indicating that while they were able to tolerate this salinity for a brief time, a limit to salinity acclimation may exist at this level.

Although oxygen consumption rate decreased with salinity up to 95 g/l, it was only sufficient in maintaining osmotic balance up to approximately 65 g/l salinity, beyond which tilapia increased drinking rate and osmoregulatory capacity. However, because reductions in \(\dot{M}O_2\) were also time dependent, this cannot be the sole mechanism to explain the salinity tolerance of this species. Kültz and Onken (1993) described the opercular epithelium as thickened with reduced conductance after a 5 week exposure to 60 g/l. The conductance of the epithelium was dramatically reduced, and as a result, osmotic water loss and diffusive ion gains were held to a minimum under normal blood flow conditions. Ion excretion capacity of MRCs was reduced, but the number of MRCs increased to such a degree that short circuit current (\(I_{sc}\); equivalent to the secretion rate of
CI) was maintained. The metabolic adjustments observed in chapters 2 and 3 may be a temporary mechanism that allows tilapia to tolerate a hypersaline environment while the conductance of the branchial epithelium is adjusted, as described by Kültz and Onken (1993). While a time course for this restructuring is highly speculative, data from chapter 3 indicate that it may take place at any time after 14 d, but before 35 d (Kültz and Onken, 1993).

Energetics of Osmoregulation

Numerous studies have been attempted in order to gain insight into the energy demands of osmoregulation (De Boeck et al., 2000; Febry and Lutz, 1987; Morgan and Iwama, 1999). Estimates of osmoregulation have ranged from as high as half the organisms metabolic rate (Kirschner, 1995) to as low as 2-4% of metabolic rate (Morgan and Iwama, 1999). Given that metabolic rate is negatively correlated with branchial NKA in these studies, it seems that the energy budget for tilapia osmoregulation favors the latter argument. While the maintenance of osmoregulation may not be as energetically expensive as has been hypothesized, the cost of switching from hyper- to hypoosmoregulation appears to be quite high. Morgan et al. (1997) found that oxygen consumption rates after 4 d of exposure to 25 g/l salt water were significantly elevated in Mozambique tilapia relative to fish in isosmotic or fresh water. The peak in consumption coincided with increased Na\(^+\), K\(^+\)-ATPase activity and growth hormone levels, and it was hypothesized that 20% of metabolic energy was involved with osmoregulation in tilapia after 4 d of exposure. In the experiment presented in chapter 3, metabolic rate was highest 5 d after fish were transferred from FW to 15 g/l; while the
magnitude of transfer was less than the Morgan et al. (1997) protocol; it represents a
switch between a FW and SW-acclimated animal. \( \dot{M} \frac{O_2}{O_2} \) then declined to a relatively
stable rate at 14 and 28 d in 15 g/l-acclimated fish. In contrast, once the fish were
hypoosmoregulatory, and exposed to 75 g/l, branchial NKA activity was 800% of the
value observed when \( \dot{M} \frac{O_2}{O_2} \) peaked at 15 g/l after 5d (during FW-SW switch), yet \( \dot{M} \frac{O_2}{O_2} \) at
75 g/l was reduced by 32% relative to those fish. These data indirectly suggest that the
high costs of osmoregulation when FW fishes are transferred to SW are not likely NKA
activity-mediated, but result from the restructuring involved with becoming SW
acclimated. Once the epithelium is SW-acclimated, the energy required to power
branchial NKA appears to be a very small portion of whole animal metabolic rate relative
to liver ATPase activity. When determining the costs of osmoregulation by isolated gills
in FW and SW-acclimated cutthroat trout (\textit{Oncorhynchus clarki clarki}) the energetic cost
of NaCl transport was found to be <4% of the animals total energy budget (Morgan and
Iwama, 1999), which appears likely in the case of hypersaline-exposed Mozambique
tilapia hybrid.

\textbf{The Effects of Temperature on Salinity Tolerance}

As mentioned above, metabolic adjustments may play a central role in the salinity
tolerance of this species; if this is the case, an increase in temperature would certainly
interfere with salinity tolerance, in particular during acute changes in temperature. In
longer-term exposures tilapia showed signs of acclimation following transfer to 35°C, and
eventually regained osmotic control after 14 d. Although plasma osmolality was similar
in 25 versus 35°C acclimated SW tilapia, there was no difference in NKA activity, NKA
per cell, or MRC size between the two groups. Exposure to a 10°C decrease in temperature has a much more pronounced effect on this species; Exposure to 15°C consistently disrupted osmotic balance in all salinities. Although considered to be a warm water eurythermal species, 15°C was commonly used throughout my experiments, as it is within the range of temperatures that tilapia are exposed to in the Salton Sea (Watts et al., 2001). Through reduction in metabolism, cold temperature may partially alleviate the osmotic gradient across the gill; however the decrease in enzyme kinetics appears to outweigh that benefit. NKA activity was markedly decreased regardless of acclimation temperature when assayed at 15°C. In short term protocols, death and ecological death (loss of orientation) were commonly observed at this temperature at a range of salinities (35-60 g/l). However, when SW-acclimated fish held at 25°C were transferred to 15°C for two weeks, there were signs of acclimation. TMA analysis showed an MRC hypertrophy following 2 weeks of acclimation to 15°C, and renal NKA content was also increased; however, neither of these responses was sufficient to restore plasma osmolality values to the levels measured at 25 or 35°C. Based on the responses of tilapia to cold temperatures in hypersaline water, the combined stress of high salinity and fluctuating temperature should not be ruled out as a potential cause for the wide scale mortality events that are unique to tilapia within the winter months at the Salton Sea.

Summary

New physiological discoveries were made with these experiments, including the first evidence of a biochemical basis for salinity-based reduction in whole animal metabolic rate, and the first direct measurement of greater NKA abundance within MRCs
of a SW-acclimated fish. Within the ecological framework of the Salton Sea, experimental results from laboratory studies strongly implicate the interaction between salinity and temperature as a cause for the large-scale fish kills that have plagued the fishery. This species has an impressive salinity tolerance when exposed to temperatures within its preferred thermal range; however, when temperature is reduced, that salinity tolerance is dramatically reduced.
References


APPENDIX I: Justification for use of Instant Ocean in salinity challenge experiments representing conditions in the Salton Sea

An experiment was conducted in order to test for differences in osmoregulatory ability in tilapia exposed to salinities with varying calcium and sulphate concentrations (see Table A.2 for comparisons). We acclimated tilapia to 1086 mOsm, representing the osmolality of natural Salton Sea water at the time this experiment was conducted (Spring 2003), in three water types Salton Sea water (SS) Instant Ocean (IO), and Instant Ocean containing the appropriate levels of Ca\(^{2+}\), Mg\(^{2+}\), and SO\(_4\)\(^{2-}\) making them equivalent to the Salton Sea (Instant Salton Sea; ISS). Following acclimation to 1086 mOsm at 25°C, fish were transferred to 15 or 35°C for five days in the respective ion compositions.

At 24 and 120 h following transfer to 15 or 35°C, seven tilapia were killed by lethal dose of Benzocaine dissolved in ethanol and diluted to a final concentration of 0.7 g/l. The caudal peduncle was severed and blood was collected into heparinized microhematocrit centrifuge tubes, and spun in a Damon IEC MB microhematocrit centrifuge. Tubes were broken and the plasma fraction was collected and analyzed for plasma osmolality using a Wescor 5520 vapor-pressure osmometer (Wescor Inc. Logan, Utah, USA).

Results are presented as the mean for each acclimation tank with n=7, and analyzed by one way ANOVA using SigmaStat version 3.0.
Table A.1. Comparisons of the major ion concentrations in Salton Sea (SS), Instant Ocean water (IO), and Instant Salton Sea water (ISS); ionic concentrations are presented in grams per liter. Units of osmolality are mOsm/kg H₂O.

<table>
<thead>
<tr>
<th>Ion</th>
<th>SS</th>
<th>IO(^a)</th>
<th>ISS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td>1.34</td>
<td>1.45</td>
<td>1.34</td>
</tr>
<tr>
<td>K</td>
<td>0.281</td>
<td>0.415</td>
<td>0.348</td>
</tr>
<tr>
<td>Ca</td>
<td>1.11</td>
<td>0.423</td>
<td>1.11</td>
</tr>
<tr>
<td>Na</td>
<td>12.7</td>
<td>12.2</td>
<td>12.7</td>
</tr>
<tr>
<td>SO₄</td>
<td>10.3</td>
<td>2.54</td>
<td>10.3</td>
</tr>
<tr>
<td>Cl</td>
<td>17.8</td>
<td>21.2</td>
<td>17.8</td>
</tr>
<tr>
<td>ppt</td>
<td>43</td>
<td>38</td>
<td>43</td>
</tr>
<tr>
<td>mOsm</td>
<td>1086</td>
<td>1086</td>
<td>1086</td>
</tr>
</tbody>
</table>

\(a\): taken from Atkinson & Bingman (1998)

Results and Conclusions

Comparing the water types at 1086 mOsm at 25°C, there was a small increase in plasma osmolality within the group of fish exposed to ISS (p<0.05; Fig. A.1), however, values from all three groups were well within a normal homeostatic range for this animal (see chapters 2 and 3). When fish acclimated to 1086 mOsm were transferred to 35°C there was a similar pattern seen in plasma osmolality; after five days, it was slightly but significantly increased in all three water types (Fig. A.1). When transferred to 15°C, there was 100% mortality following a dramatic increase in plasma osmolality in the SS group, while in the IO and IS groups, there was little change. These data indicate that the total osmolality of the water, rather than the specific ion concentrations had the greatest effect on hypoosmoregulation in tilapia. However, under the more stressful condition of a ±10°C temperature change, some effects of the different water types became apparent. There was substantial osmoregulatory stress and mortality in natural SS water following a 10°C decrease in temperature, while there was no mortality, and only minor ionoregulatory disturbances in the other water treatments.
At a similar osmolality (1086 mOsm), there were higher total dissolved solutes in the SS and ISS waters versus IO water (43 versus 38 g/l, respectively; table A.12), yet there was little difference between the patterns of change in plasma osmolality following temperature challenge in ISS relative to IO water. In contrast, the SS water, although similar in ionic composition to ISS, had substantial effects when tilapia were exposed to cold temperature, as there was a substantial loss of osmotic balance at 24 h, and 100% mortality within 48 h of transfer. Clearly the large effects of a reduction in temperature on osmoregulation and survival in SS water cannot be related specifically to the relative elevation of Ca$^{2+}$ and SO$_4^{-}$ levels and must be related to other factors, which remain unknown. From these results it is reasonable to conclude that the use of natural Salton Sea water in physiological experiments should be avoided.
Figure A.1. Mean plasma osmolality (±SE) in tilapia hybrids acclimated to 43 g/l salinity and transferred to 15 or 35°C in a) Salton Sea water (15°C, p=0.002, F=20.25; 35°C, p<0.001, F=36.13), b) Instant Ocean Water (15°C, p<0.001, F=13.40; 35°C, p<0.001, F=21.15), and c) Instant Salton Sea water (15°C, p=0.09, F=2.71; 35°C, p<0.001, F=8.10). * denotes significant differences from time 0 (25°C).