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The effect of temperature on juvenile Mozambique tilapia hybrids (*Oreochromis mossambicus* x *O. urolepis hornorum*) exposed to full-strength and hypersaline seawater

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Abstract

The effects of temperature on the salinity tolerance of Mozambique–Wami tilapia hybrids (*Oreochromis mossambicus* x *O. urolepis hornorum*) were investigated by transferring 35 g/l, 25 °C-acclimated fish to 35, 43, 51 or 60 g/l salinity at 15, 25 or 35 °C for 24 h, and by assaying gill tissue for branchial Na^+, K^+-ATPase activity at the three temperatures after acclimating the fish to 15, 25 or 35 °C for 2 weeks. Tilapia survived all salinities at 25 and 35 °C; however, at 15 °C, mortality was 85.7% and 100% in the 51 g/l and 60 g/l groups, respectively. There was a significant interaction between temperature and salinity, as plasma osmolality, $[Na^+]$ and $[Cl^-]$ were significantly increased at 51 and 60 g/l salinity in 35 °C water ($P<0.001$). Additionally, muscle water content was significantly reduced at 43 g/l, 15 °C relative to pre-transfer values ($P<0.001$). Branchial Na^+, K^+-ATPase activity was reduced at 15 °C regardless of acclimation temperature, and 25 °C-acclimated gill tissue did not show an increase in activity when assayed at 35 °C. Results indicate that the effects of a combined temperature–salinity transfer on plasma osmolality and ion concentrations, as well as muscle water content, are greater than when either challenge is given alone. Additionally, branchial Na^+, K^+-ATPase activity is altered when assayed at varying temperatures; in the case of 15 °C, regardless of acclimation temperature. Our enzyme activity data may indicate the presence of a high temperature isoform of branchial Na^+, K^+-ATPase enzyme.

Keywords: Temperature; Salinity acclimation; Tilapia; *Oreochromis mossambicus*; 24 h salinity challenge; Hypersalinity; Na^+, K^+-ATPase

1. 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 are often regarded as one of the most saline tolerant teleosts. Surprisingly, however, there are very few studies investigating the physiological mechanisms associated with transfer to salinities greater than seawater (Kultz and Jurss, 1993; Kultz

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and Onken, 1993). It also has been reported that temperature influences the osmoregulatory ability of fishes, where a reduction in temperature below an optimal value appears to induce 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 stenothermal, with 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.

A Mozambique–Wami tilapia hybrid (*O. mossambicus* x *O. urolepis hornorum*), which has also been referred to as the 'California' Mozambique tilapia, currently resides in southern California (Costa-Pierce and Doyle, 1997). Living within drainage ditches, creeks, aquaculture farms, and the Salton Sea, this hybrid tilapia is dispersed over 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 tilapia hybrids. 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 sub-lethal indicators of osmoregulatory stress. In salmonids, 24 h seawater challenge tests are routinely used to assess the hypo-osmoregulatory ability of smolts prior to their release from hatcheries (Clarke and Blackburn, 1977; Wedemeyer et al., 1980; Blackburn and Clarke, 1987). Fish are directly transferred from freshwater to full-strength seawater, and following 24 h, 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. Additionally, 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.

In addition, 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.

2. Materials and methods

2.1. Fish

Pacific Aquafarms in Niland, CA, USA, donated 300 juvenile tilapia hybrids (*O. 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, 10–20 and 20–35 g/l) in a 440 l filtered, aerated, tank with 4 days allowed for acclimation at each salinity. Fish were then left for 2 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.

2.2. Series I: 24 h hypersaline challenges

Salinity challenges were conducted in 60 l glass aquaria; static systems with aeration and filtration, with Siporex biofilters used in all treatments. Fish were subjected to a 24 h salinity challenge by direct transfer of seven fish 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 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 H_2O, 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 3 min 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 540 nm; [Hb] was then converted to mean cell hemoglobin concentration (MCHC), calculated by [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 h at 70 °C.

Plasma osmolality was measured using a Wescor 5500 V.P. osmometer, and expressed as mOsm/kg H_2O. 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).

2.3. Series II: 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 2 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 Na_2, 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 micromoles of ADP per hour per milligram total protein; protein was determined using the biuret reagent.

2.4. 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. Additionally, regression analysis was used to investigate correlation between plasma osmolality, muscle water content and external salinity. 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.).

3. Results

3.1. Series I: 24 h hypersaline 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 g/l 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 lies 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. 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. 1b, c). 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. 1a). Muscle water content showed a small significant interaction between temperature and salinity ($P=0.047$) (Fig. 2). Finally, no significant differences were observed in Hct or MCHC values

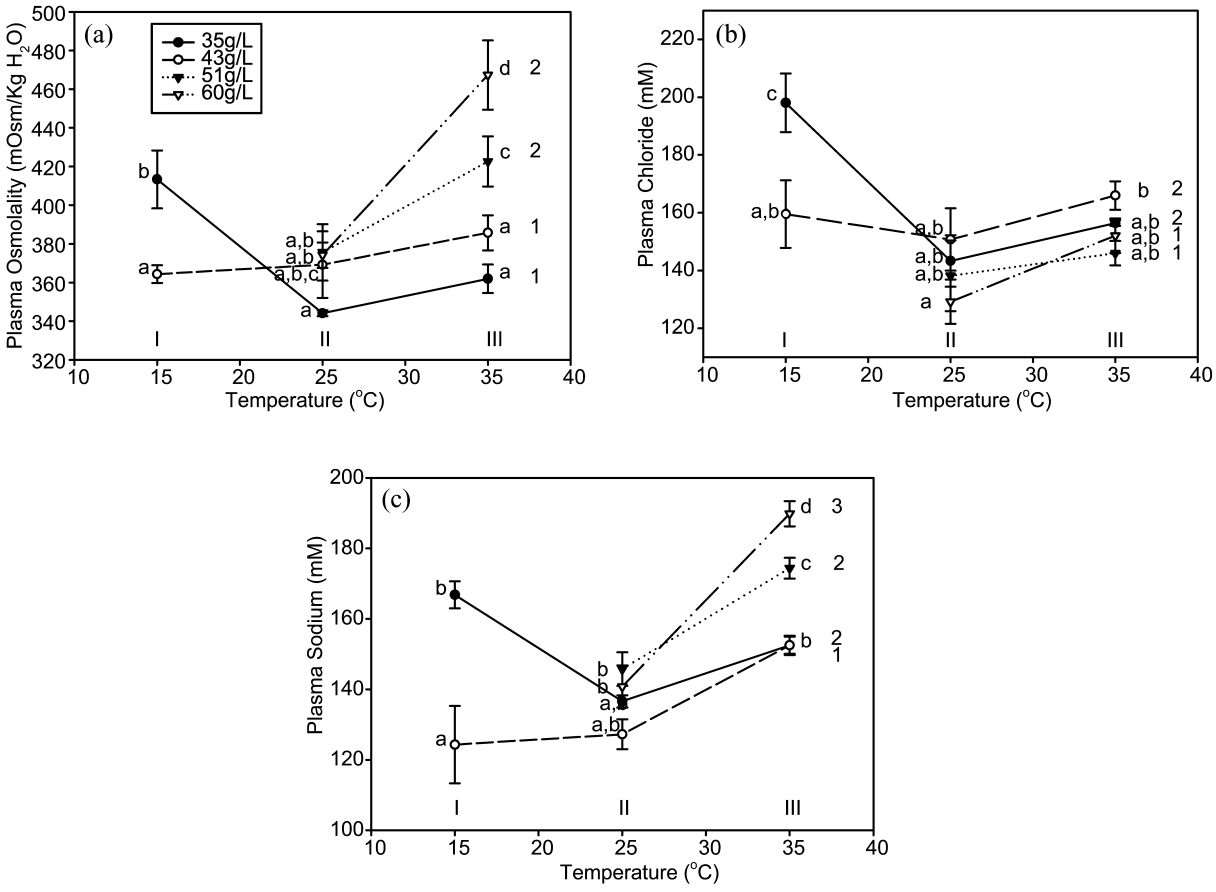

Fig. 1. The effect of salinity (35, 43, 51 and 60 g/l) and temperature (15, 25 and 35 °C) 24 h following transfer on (a) plasma osmolality, (b) plasma chloride, and (c) plasma sodium in Mozambique–Wami tilapia hybrids. Roman numerals (I, II, III) indicate significant effects of temperature, Arabic numbers (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 60 g/l at 15 °C. Error bars indicate S.E.M. ($n=7$).

(pooled values = 24.1 ± 0.10 mM and 2.84 ± 0.12 mM, respectively; data not shown).

The data obtained for plasma osmolality and muscle water content were re-interpreted using regression analysis. Analysis of mean plasma osmolality with salinity revealed a weak relationship at 25 °C, and a sharp increase with salinity at 35 °C ($P=0.02$ $r^2=0.310$, and $P=0.031$ $r^2=0.939$, respectively) (Fig. 3). Regression analysis of mean muscle water content with salinity revealed a negative linear correlation at 25 °C ($P<0.0001$ $r^2=0.9924$), while at 35 °C, muscle water content showed a slightly positive linear correlation ($P=0.0005$, $r^2=0.801$) (Fig. 4). Regressions were not conducted for data at 15 °C due to high mortality and a limited data set.

3.2. Series II: the effect of 2-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. 5). In fish acclimated to 25 and 35 °C for 2 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

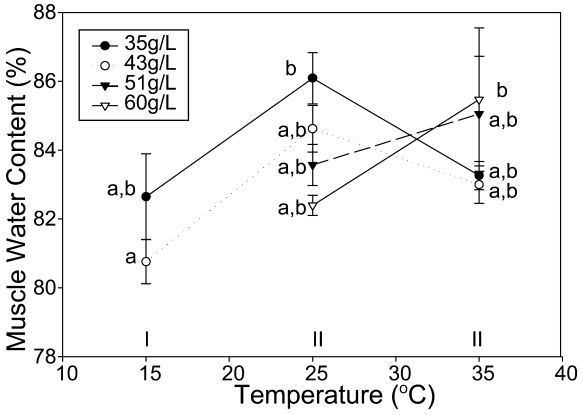

Fig. 2. The effect of simultaneous salinity (35, 43, 51 and 60 g/l) and temperature (15, 25 and 35 °C) transfer on muscle water content in Mozambique–Wami tilapia hybrids following 24 h. Due to mortality, no data are given for 51 or 60 g/l at 15 °C. See legend of Fig. 1 for further details.

increase in activity when assayed at 35 °C (Fig. 5).

4. Discussion

4.1. Effect of combined temperature and salinity challenges

The objective of this study was to assess the acute effects of a 24 h hypersaline challenge at

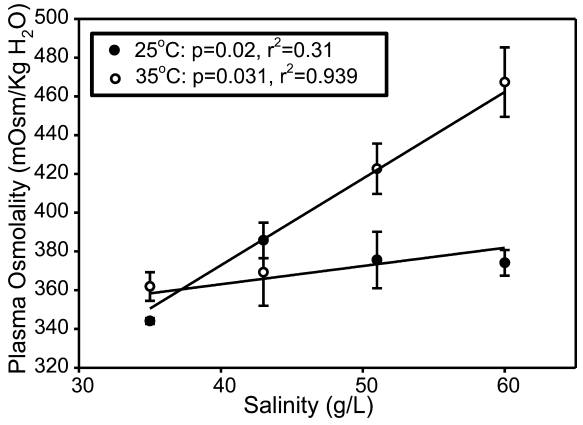

Fig. 3. Regression analysis between salinity and plasma osmolality 24 h following simultaneous salinity (35, 43, 51 and 60 g/l) and temperature (15, 25 and 35 °C) transfer in Mozambique–Wami tilapia hybrids. Osmolality at 25 °C showed a minimal linear increase with salinity ($P=0.0005$, $r^2=0.801$), while at 35 °C, osmolality showed a sharp linear increase ($P=0.03$, $r^2=0.9390$), and error bars indicate S.E.M. ($n=7$).

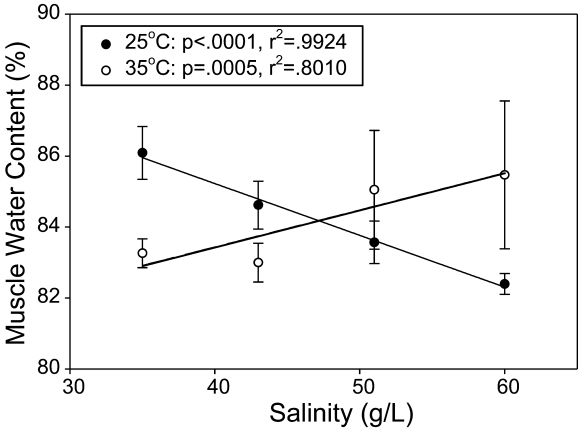

Fig. 4. Regression analysis between salinity and muscle water content 24 h following simultaneous salinity (35, 43, 51 and 60 g/l) and temperature (15, 25 and 35 °C) transfer in Mozambique–Wami tilapia hybrids. Muscle water at 25 °C showed a negative linear relationship with salinity ($P<0.0001$, $r^2=0.992$), while at 35 °C, there is a slight increase ($P=0.0005$, $r^2=0.801$), and error bars indicate S.E.M. ($n=7$).

various temperatures. At 25 °C, tilapia plasma osmolality was well regulated 24 h after transfer to all salinities. 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

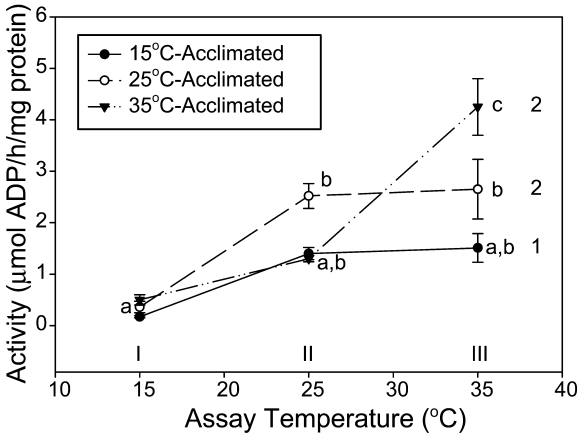

Fig. 5. In vitro branchial Na^+, K^+-ATPase activity assayed at 15, 25 and 35 °C in tissues isolated from Mozambique–Wami tilapia hybrids acclimated to 15, 25 or 35 °C 2 weeks prior. Roman numerals (I, II, III) indicate significant effects of assay temperature, Arabic numbers (1, 2, 3…) indicate significant effects of acclimation temperature, and letters indicate significant differences among individual means. Error bars indicate S.E.M. ($n=7$).

temperature (Hwang et al., 1989); however, direct transfers to salinities used in this study are not available for comparison. In the current study, when temperature was elevated or reduced by 10 °C, tilapia hybrids no longer successfully maintained the same steady state after exposure to elevated salinity, evident by increasing osmolality with salinity at 35 °C, and high mortality at salinities greater than 47 g/l at 15 °C. Rapid transfer to elevated environmental salinity in teleosts commonly results in an increased plasma osmolality due to osmotic water loss and diffusive ion gains (Assem and Hanke, 1979; Hwang, 1987; Hwang et al., 1989; Kultz and Jurss, 1993; Dang et al., 2000; Daborn et al., 2001). Euryhaline species generally respond to these challenges with proliferation and hypertrophy of branchial chloride cells, the primary site of NaCl excretion (Foskett et al., 1981; Perry, 1997; Marshall and Bryson, 1998); however, at 24 h the contribution of changes in gill morphology are most likely minimal.

There was a negative linear correlation between muscle water content and salinity observed at 25 °C, most likely due to passive water loss resulting from increased ion influx. Loss of water from the muscle may have beneficial effects, however, as it prevents lethal dehydration in the cardiovascular system, and allows for survival while salt excretion mechanisms are upregulated. The maintenance of plasma osmolality at 25 °C across the range of salinities investigated may reflect this. Muscle water content in tilapia exposed to 35 °C remained constant. The cause for this remains unclear, as Handeland et al. (2000) observed that muscle water decreases were more significant in Atlantic salmon (*Salmo salar*) when transferred to 18.9 relative to 9.1 or 14.4 °C.

4.2. Mechanisms of physiological impairment

High mortality of tilapia hybrids exposed to salinities above 47 g/l at 15 °C is most likely due to osmoregulatory stress (Al Amoudi et al., 1996). Low temperatures 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).

Surprisingly, plasma osmolality, and $[Na^+]$ were significantly higher in 35 g/l- vs. 43 g/l-exposed fish at 15 °C. In a past study, Mozambique tilapia exposed to 45 and 60 g/l had significantly decreased chloride-accessory cell leaky junction conductance, which benefit the fish by reducing passive ion gains, and thus, ion regulatory costs (Kultz and Onken, 1993; Sardella et al., unpublished data).

Osmoregulatory disturbances at 15 and 35 °C may also result 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, caused by altered lipid structure, that were responsible for increased water and ion permeability (Johnston and Cheverie, 1985). Membrane disruptions such as these may also explain the increase in muscle water at high temperature. 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).

Teleosts transferred to hyperthermic water experience an elevated oxygen demand due to increased metabolic rate; as a result, cardiac output tends to correlate positively with temperature (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 (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). In the current study, these changes may be reflected by the steady increase of plasma osmolality with salinity, and elevated $[Na^+]$ at 35 °C. 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 $[Na^+]$ are much greater than those observed for $[Cl^-]$. Fish transferred to more saline water experience 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 $[Na^+]$, decrease in $[Cl^-]$, or a combi-

nation of the two. The relatively minor effect of salinity transfer on plasma [Cl^-] and the large effect on [Na^+] is likely indicative of blood pH compensation associated with hypersaline exposure.

Acclimation of fish to 15 °C for 2 weeks did not alter in vitro Na^+, 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 exposed to 4.1 °C, in part, to a reduced Na^+, K^+-ATPase activity in response to lowered enzyme kinetics. 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 is ability to up regulate enzyme activity, at least up to 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 fresh water- vs. seawater-acclimated fish (Lee et al., 1998), based on the results in the current study, isoform switching between fish exposed to different acclimation temperatures may also occur, and is worthy of further investigation. Furthermore, it has been hypothesized that an upper temperature limit to maximal Na^+, K^+-ATPase activity may exist (Kieffer and Tufts, 1996). This hypothesis is consistent with current findings from 15 and 25 °C-acclimated fish, and indicates that maximal activity limits may also be isoform-specific.

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.

5. Conclusions

Based upon mortality and sub-lethal indicators of osmoregulatory stress during transfer to increased salinity, this study clearly demonstrates 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 indicate that osmoregulatory ability may be compromised when tilapia hybrids are 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; an experiment involving longer exposure durations may better reveal temperature limitations on recovery following transfers to increased salinity.

Additionally, decreased temperature results in depression of Na^+, K^+-ATPase activity, regardless of acclimation temperature, and in 25 °C-acclimated gills, an upper limit to maximal activity may exist.

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