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COLD TEMPERATURE-INDUCED OSMOREGULATORY FAILURE: THE PHYSIOLOGICAL BASIS FOR TILAPIA WINTER MORTALITY IN THE SALTON SEA?

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ABSTRACT

The Salton Sea is a large saline lake in southeastern California that has been plagued by large-scale mortality among the fishes that once comprised a robust recreational fishery. In addition to very high salinity (43-47 g/l), other stressors such as large fluctuations in temperature exist that when combined with salinity can result in a severe physiological challenge to resident fishes. Fish kills during winter months are predominantly comprised of 'California' Mozambique tilapia, *Oreochromis mossambicus* x *O. urolepis hornorum*, so we investigated the effects of temperature and salinity interaction as a cause of the mortality. Tilapia acclimated to 43 g/l were transferred from 25 ºC to 15 or 35 ºC in order to assess the effect on osmoregulatory ability. There were significant increases in plasma osmolality and [Na^+] 24 h after tilapia were transferred to 15ºC that recovered to pre-transfer values by 120 h. In order to demonstrate the additive effects of temperature and salinity, we transferred tilapia from 35 g/l salinity at 25 ºC to 43 g/l salinity at 15, 25, and 35 ºC. The combined temperature and salinity challenge significantly affected plasma osmolality and branchial Na^+, K^+-ATPase (NKA) activity, with fish in the 15 ºC group experiencing mortality after 12 h. Branchial NKA activity was significantly increased 3 h following transfer, but was reduced by 6 h in the 15 and 35 ºC groups. The ability of this species to effectively osmoregulate as well as tolerate salinity transfer was impaired following a reduction in ambient temperature. Based on these experimental findings, we concluded that cold-induced tilapia mortality during winter months at the Salton Sea is due to osmoregulatory failure.

INTRODUCTION

The Salton Sea is a large (980 km^2) inland lake that was created in the early 20th century when water from the Colorado River flooded the Imperial Valley, forming a large 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 its physiologically-challenging environment, the Salton Sea has supported a substantial fish population for decades. Over 30 species

have been introduced into the Sea during the last 80 years; however, only orangemouth corvina, *Cynoscion xanthulus*, bairdiella, *Bairdiella icista*, and sargo, *Anisotremus davidsoni*, have flourished since the mid-1950s (Walker et al. 1961). The dominant fish species since the mid-1960s has been a Mozambique tilapia hybrid, *Oreochromis mossambicus* x *O. urolepis hornorum*, which is also referred to as the California Mozambique tilapia (Costa-Pierce and Doyle 1997). Together these four species comprise a large portion of the total Salton Sea fishery, and have all been shown to tolerate high salinities in laboratory studies (Hanson 1970; Sardella et al. 2004b). However, there are other stressors playing deleterious synergistic roles in the Salton Sea, including a large seasonal temperature range (12-35ºC), high [SH^-] (up to 5 mg/l), high [NH_3] (1.2 mg/l), toxic metalloids such as selenium and arsenic, low dissolved oxygen levels with occasional anoxia, and various disease outbreaks (Gonzalez et al. 1998; Watts et al. 2001; Riedel et al. 2002). The continual salinity increase of the Salton Sea is a major concern for conservation agencies such as the Salton Sea Authority. Fish kills occur there throughout the year, with a peak in mortality rate typically during the hot summer months. Unlike corvina, croaker, and sargo, tilapia are also subject to a high rate of mortality during the winter (Hurlbert et al. 2007), which may result from a low tolerance for cold temperatures (Al Amoudi et al. 1996, Sardella et al. 2004a). While summer mortality is typically attributed to low oxygen availability and high [SH^-] (Watts et al. 2001; Hurlbert et al. 2007), the etiology of the winter tilapia kills has yet to be fully elucidated.

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). This impressive salinity tolerance has also been documented in pure Mozambique tilapia (Stickney 1986), as has this species inability to tolerate cold ambient temperatures (Al Amoudi et al. 1996). The Salton Sea has a wide annual temperature range (12 - 35 °C), and regularly windy conditions can result in a complete turnover of the lake, making it difficult for fish to avoid temperature extremes (Watts et al. 2001). In the few studies conducted to date, changes in temperature have been shown to have a negative effect on the salinity tolerance of fishes (Al Amoudi et al. 1996, Allanson and Bok 1971, Staurnes et al. 2001). Tilapia are considered eurythermal, but their survival has been reduced when they are exposed to water temperatures that are representative of the Salton Sea during the winter (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 negatively affect osmoregulation (Al Amoudi et al. 1996, Robertson and Hazel 1999, Almansa et al. 2003). We hypothesize that temperature-induced tilapia mortality during winter months at the Salton Sea results from osmoregulatory failure.

The purpose of this study was to determine 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) at 25 ºC before transfer to 15 or 35 ºC 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. A second experiment was carried out in order to demonstrate how temperature can negatively affect what is a mild salinity challenge for this species (Sardella et al. 2004b). In this experiment fish were acclimated to SW (33 g/l) at 25 ºC and then transferred to 43 g/l at 15, 25, or 35 ºC. 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 reared in freshwater and acclimated to 35 g/l (SW) gradually over 3 weeks (as described by 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 ºC. Fish used in both experiments weighed on average 30.18 ± 0.75 g. Salinity was manipulated by adding Instant Ocean synthetic sea salts[1], and measured by light refractometery.

Experiment 1: Temperature Transfer

Thirty-five SW-acclimated tilapia were acclimated to a salinity of 43 g/l at 25 ºC and for 2 weeks. Following this acclimation temperature was altered from 25 ºC to 15 or 35 ºC over one approximately hour. Seven fish were sampled prior to transfer, and seven fish were sampled (see below) at each temperature 24 and 120 h following temperature change.

Experiment 2: Temperature Transfer during Simultaneous Salinity Transfer

After 3 weeks of acclimation to SW at 25ºC, seven CA Mozambique tilapia were sampled. Twenty-eight SW-acclimated tilapia were directly transferred from SW at 25ºC to 43 g/l at 25 ºC, with seven fish being removed and sampled (see below) at 3, 6, 12, and 24 h following transfer. This transfer and sampling protocol was then repeated with transfers from SW at 25 ºC to 43 g/l at either 15 or 35 ºC.

[1]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 contains approximately two-fold calcium ion and three-fold sulphate ion. In previous experiments (Sardella 2006), it was shown the effects of these differences were negligible with respect to the osmoregulatory ability of tilapia.

Sampling

Fish were sacrificed 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 Microcentrifuge 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 ºC oven. The difference in weight was expressed as a percentage of original wet weight. Lastly, the second and third left gill arches were collected, frozen quickly on dry ice, and stored at -80 ºC for later analysis of NKA activity.

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, deoxycholic acid (0.05%). Branchial Na^+, K^+-ATPase activity was determined as described by McCormick (1993) and expressed as µMol of ADP per hour per µg total protein. Protein was measured using the Biuret method.

Statistical Analyses

A two-way analysis of variance (ANOVA) test was used to determine the effects of temperature and time of exposure on all parameters. A post hoc Holm-Sidak multiple means comparison test was performed when two-way ANOVA results were significant. Statistical tests were performed using SigmaStat version 3.0, with an α value of 0.05 (n = 7).

RESULTS

Experiment 1: Temperature Transfer

In 43 g/l salinity-acclimated fish, transfer to 15 or 35 ºC did not result in loss of orientation or mortality but several sub-lethal effects were observed. Two-way ANOVA results for plasma osmolality, [Na^+], and [Cl^-] following transfer are presented in Tables 1-3. Both osmolality and [Na^+] were significantly increased at 24 h in 15 ºC water, and osmolality was reduced to near pre-transfer values after 120 h (Fig. 1a). In contrast, plasma [Na^+] decreased from the peak value at 24 h to an elevated but stable level (Fig. 1b). Plasma osmolality in 35 ºC-exposed fish was significantly increased 120

Table 1. Two-way ANOVA results for plasma osmolality following temperature transfer.

Factor	DF	SS	F	*P*
Time	2	15161.5	18.76	< 0.001
Temperature	1	1620.7	4.01	0.054
Interaction	2	7529.1	9.31	< 0.001
Residual	32	12932.7		
Total	37	36436.7		

Table 2. Two-way ANOVA results for plasma chloride following temperature transfer.

Factor	DF	SS	F	*P*
Time	2	3217.5	59.68	< 0.001
Temperature	1	416.4	15.40	< 0.001
Interaction	2	496.5	9.20	< 0.001
Residual	33	889.5		
Total	38	4863.5		

Table 3. Two-way ANOVA results for plasma chloride following temperature transfer.

Factor	DF	SS	F	*P*
Time	2	699.11	8.28	0.001
Temperature	1	186.72	4.28	0.047
Interaction	2	160.92	1.91	0.165
Residual	33	1349.99		
Total	38	2351.24		

h following transfer, but plasma [Na^+] was significantly increased at both 24 and 120 h. Two-way ANOVA results for muscle water content are presented in Table 4. Muscle water content was reduced 24 h following transfer and recovered by 120 h (Fig. 2), but there was no difference between temperatures. Lastly, there were no significant changes in branchial NKA activity at 24 or 120 h following temperature transfers (Pooled Value = 7.69 ± 0.62 µmol/h/µg protein; data not shown).

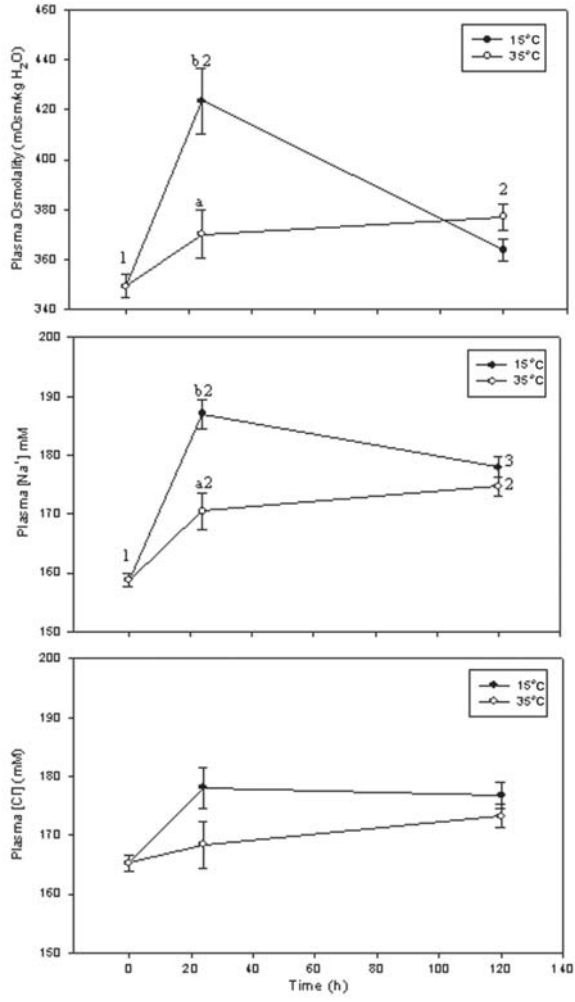

Figure 1. The effect of a ±10 ºC temperature change on a) plasma osmolality, b) plasma [Na^+], and c) plasma [Cl^-] in California Mozambique tilapia acclimated to 43 g/l salinity. Letters indicate significant differences between fish at 15 ºC compared to 35 ºC at a given time, whereas numbers denote significant differences relative to time zero within a given temperature as determined by two-way ANOVA ($p<0.001$).

Experiment 2: Temperature Transfer during Simultaneous Salinity Transfer

There was no mortality or loss of equilibrium in tilapia transferred from SW at 25 ºC to 43 g/l salinity at 25 or 35 ºC. However, when fish were transferred to 43 g/l at 15 ºC, there was 100% mortality after 12 h. Two-way ANOVA results for plasma osmolality following a simultaneous salinity and temperature transfer are presented in Table 5. In

Table 4. Two-way ANOVA results for muscle water content following temperature transfer.

Factor	DF	SS	F	*P*
Time	2	102.38	6.132	0.005
Temperature	1	15.48	1.855	0.182
Interaction	2	8.96	0.537	0.590
Residual	34	283.86		
Total	39	415.21		

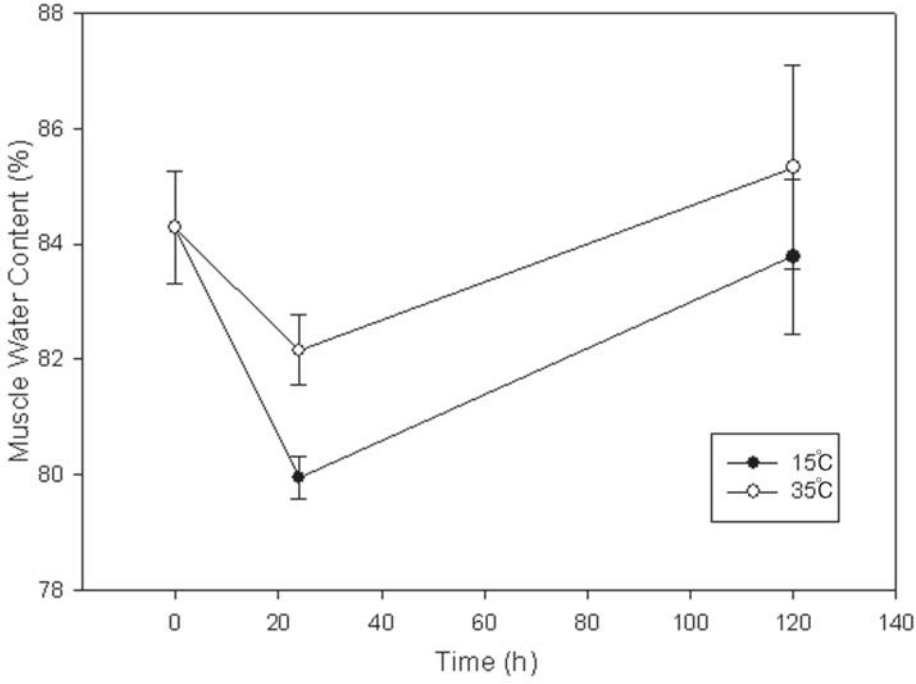

Figure 2. The effect of a ±10 ºC temperature change on muscle water content in California Mozambique tilapia acclimated to 43 g/l salinity. There was a significant effect of time as determined by two-way ANOVA ($p<0.01$).

Table 5. Two-way ANOVA results for plasma osmolality following salinity and temperature transfer.

Factor	DF	SS	F	*P*
Time	3	358081	127.9	< 0.001
Temperature	2	47440	25.43	< 0.001
Interaction	6	568235	101.5	< 0.001
Residual	54	59712		
Total	75	1096386		

fish transferred to 15 ºC, plasma osmolality increased at each time point up to 12 h, while osmolality in fish at 35 ºC was significant increased at 6 h after transfer and remained elevated over 24 h. In contrast, fish transferred to 25 ºC maintained plasma osmolality over the entire experiment (Fig. 3).

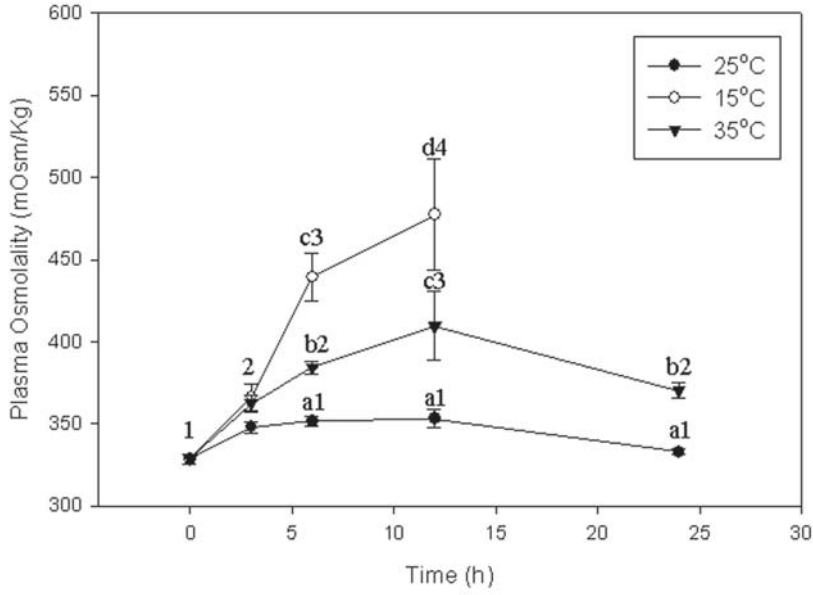

Figure 3. The effect of transfer from 35 to 43 g/l salinity at 15, 25, and 35 ºC on plasma osmolality in California Mozambique tilapia. Letters indicate significant differences due to temperature at a given time, whereas numbers denote significant differences relative to time zero within a given temperature as determined by two-way ANOVA ($p<0.001$).

Two-way ANOVA results for branchial NKA activity following simultaneous salinity and temperature transfer are presented in Table 6. NKA activity from tilapia transferred at 25 ºC was elevated at 3 h and remained high at 12 h before beginning to decline by 24 h (Fig. 4). The fish transferred at 15 and 35 ºC also showed an increase in NKA activity at 3 h but in these groups, it decreased to pre-transfer levels by 6 h. While NKA from 15 ºC-acclimated tilapia remained low up until the death of the fish, NKA activity in the 35 ºC-acclimated fish showed a second increase that was significantly higher than pre-transfer values by 24 h.

Table 6. Two-way ANOVA results for branchial NKA activity following salinity and temperature transfer.

Factor	DF	SS	F	*P*
Time	4	8.6	5.96	< 0.001
Temperature	2	12.66	17.54	< 0.001
Interaction	8	22.25	7.71	< 0.001
Residual	86	31.03		
Total	100	77.80		

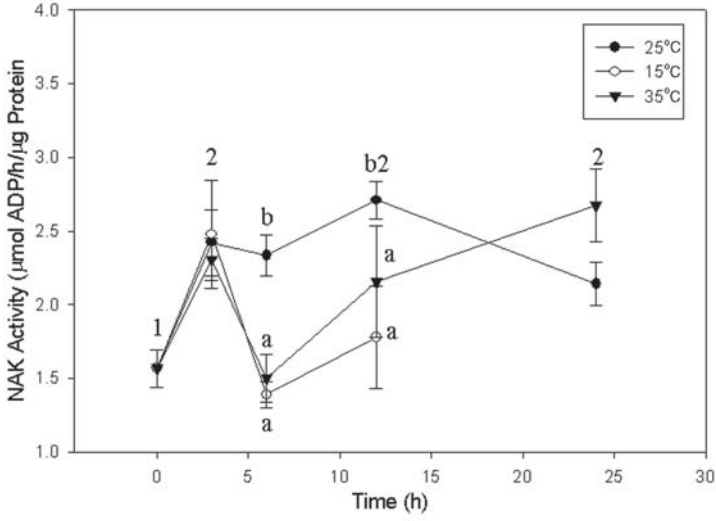

Figure 4. The effect of transfer from 35 to 43 g/l salinity at 15, 25, and 35 ºC on branchial NKA activity in California Mozambique tilapia; all assays were carried out at 25 ºC. Letters indicate significant differences due to temperature at a given time, whereas numbers denote significant differences relative to time zero within a given temperature as determined by two-way ANOVA ($p<0.01$).

DISCUSSION

All Salton Sea fish are subject to mortality events but during the winter months, the bulk of the fish kills has been tilapia (Hurlbert et al. 2007). Based on the results of this study, the effects of reduced temperature on osmoregulatory ability is potentially a large contributor to the high winter mortality. Our results agree with previously published work showing that the effects of the interaction between temperature and salinity pose a greater challenge than the application of either stress alone (Al Amoudi et al. 1996, Allanson and Bok 1971, Sardella et al. 2004a).

The goal of experiment 1 was to demonstrate the effect of a temperature transfer carried out at a constant salinity. The response of tilapia to the temperature change was similar to what has been observed following a salinity increase from 35 to 60 g/l at 25 ºC, with large increases in both plasma osmolality and [Na^+] (Sardella et al. 2004b; Fig. 1). These changes indicate an immediate loss of osmotic balance following the transfer. The increased osmolality was correlated with decreased muscle water content at 24 h (Fig. 2), as the high osmotic pressure draws water out of the intracellular compartment. Muscle water loss in this species does not occur following salinity increases at 25 ºC, even when salinities are as high as 95 g/l and plasma osmolality reaches levels greater than 400 mOsm/kg H_2O (Sardella et al. 2004b). Loss of muscle water in this study indicates a potential role for temperature in the loss of cellular water, as the levels of osmolality in this study, while high, have not been shown to decrease muscle water previously. Temperature has been shown to have detrimental effects on salt and water balance 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 (Randall et al. 1972, Gonzalez and McDonald 1992, Hochachka and Somero 2002). One hundred and twenty hours following temperature transfer there was no difference in plasma osmolality between fish transferred to 15 versus 35 ºC, but the temporary disturbance at 24 h was greater in 15 ºC exposed fish, indicating that decreases in temperature are more detrimental than increases over this range. These data were consistent with previous studies with this species (Allanson and Bok 1971, Al Amoudi et al. 1996, Sardella et al. 2004a). While it appeared that tilapia were able to acclimate to the temperature changes by 120 h in this laboratory experiment, within the Salton Sea where they must actively forage and avoid predation, as well as deal with several other environmental stressors, their threshold for salinity tolerance at 15 ºC could be dramatically reduced.

Although an abrupt temperature change represents a worse-case scenario with respect to its effects on osmoregulation, we exposed tilapia to a combined temperature and salinity challenge for 24 h based on previous findings that the first 12 h are the most crucial for tilapia acclimation (Hwang 1987, Hwang et al. 1989). Acute 24 h challenges are commonly used to assess overall fitness and have been shown in other species to be well representative of osmoregulatory ability (Brauner et al. 1992, Clarke and Shelbourn 1982, Clarke and Blackburn 1977). Tilapia were able to maintain osmotic balance following a 10 g/l increase in salinity at constant temperature for up to 24 h (Fig.

3), with minimal yet significant increases in branchial NKA activity at 12 h (Fig 4). Twelve hours has been previously described as the end of the 'crisis period' for this species, which represents the timing of the peak dehydration that must be compensated for by the fish while longer-term osmoregulatory capacity is upregulated (Hwang et al. 1989). Tilapia did not maintain plasma osmolality at the same level when temperature was increased to 35 ºC; plasma osmolality increased at 3 h following transfer, peaked at 12 h, and was still significantly elevated at 24 h (Fig. 3). Again, the peak in osmolality coincided with what Hwang et al. (1989) described as the crisis period during salinity acclimation. The increased osmolality in fish held at 35 ºC relative to 25 ºC values is likely associated with the temperature-induced increase in metabolic rate (Q_{10} effects). Under these conditions, gill water and blood flow become elevated to meet the demands of metabolism, which subsequently increases the potential for osmotic disequilibration across the gill epithelium (Randall et al. 1972, Gonzalez and McDonald 1992). The enhanced osmotic gradient resulting from hypermetabolism offsets any advantage of increased gill NKA activity (Hochachka and Somero 2002, Sardella et al. 2004a).

Fish transferred from 35 g/l salinity at 25 ºC to 43 g/l salinity at 15ºC did not survive past 12 h (the crisis period). Prior to death, fish from this treatment group had steadily increasing plasma osmolality (Fig. 3). The likely cause of mortality was loss of osmoregulatory control (Allanson and Bok 1971, Al Amoudi et al. 1996), as NKA activity has been shown to be dramatically reduced when assayed at 15 ºC (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.

Tilapia in all temperatures showed an initial increase in NKA at 3 h, but the fish with the additional imposed temperature stress were unable to maintain the elevated activity (Fig. 4), which may have ultimately led to elevated plasma osmolality. A rapid activation of gill NKA has been observed in this Mozambique tilapia (Hwang et al. 1989, Sunny and Ommen 2001) as well as the common killifish (*Fundulus heteroclitus*) (Towle et al. 1977, Mancera and McCormick 2000), which is also a well known model of euryhalinity.. The cause behind the inability to sustain NKA activity in 15 and 35 ºC water is unclear and provides an interesting course for further study.

The results clearly show that a temperature change can have a profound effect on the osmoregulatory ability of California Mozambique tilapia, and that a combined temperature and salinity challenge was more stressful than either stress applied alone. Tilapia exposed to 15 ºC in this and 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 below 15 ºC (Watts et al., 2001). While temperature changes in the Salton Sea are more gradual relative to laboratory experiments, these data provide a good example off how temperature shifts can negatively affect osmoregulation. We 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 proper at 25 ºC, and when the temperature was reduced to 15 ºC, 100% mortality resulted in as little as 3 h (Sardella 2006).

The documented use of river mouth areas within the Salton Sea by tilapia during periods of low temperature (Hurlbert et al. 2007) could possibly represent an attempt to alleviate temperature-induced osmoregulatory disturbances, but direct evidence to confirm this phenomenon has yet to be collected. Further investigation into the possible selection of lower salinities when faced with a temperature stress would serve as a test of this hypothesis. Based on experimental findings from this and other studies involving abrupt temperature challenges, it is reasonable to conclude that osmoregulatory failure during the winter cold is a major contributor to tilapia mortality in the Salton Sea.

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