RESEARCH ARTICLE

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The effects of acclimation temperature, salinity, and behavior on the thermal tolerance of Mozambique tilapia (*Oreochromis mossambicus*)

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Abstract

Mozambique tilapia have been shown to be incredibly stress tolerant with respect to environmental salinity, hypoxia, and ammonia concentrations. Temperature challenges to this species have shown that they have difficulty with cold acclimation. The purpose of this study was to measure the effects of acclimation temperature and salinity on the thermal tolerance of Mozambique tilapia as assessed by critical thermal maxima (CT_{Max}) and critical thermal minima (CT_{Min}). We also monitored fish behavior and quantified ventilation rate. To our knowledge, this study was the first to investigate upper and lower thermal tolerances, and the effect of environmental salinity in this physiologically impressive species. Using predictive regression analyses of the thermal limits, thermal tolerance polygons were constructed and total areas were calculated 678.9°C^2 for freshwater (FW)-acclimated tilapia, and 739.4°C^2 seawater (SW)-acclimated tilapia. During the thermal challenges, we observed two novel behaviors in response to thermal challenge, ventilation cessation behavior (VCB) and aquatic surface respiration (ASR), and we conclude that the use of these behaviors extended the thermal limits of these fish in both FW and two-thirds SW by limiting the exposure of the gill epithelium to the changing environment.

1 | INTRODUCTION

The Mozambique tilapia (*Oreochromis mossambicus*) is one of the most environmental stress-tolerant species in the world. Previous work over many years has documented that this tilapia can survive, and acclimate to, a wide range of salinities (Heijden et al., 1997; Kamal & Mair, 2005; Kultz and Jurss, 1993; Lorenzi & Schlenk, 2014; Morgan, Sakamoto, Grau, & Iwama, 1997; Sardella, Matey, Cooper, Gonzalez, & Brauner, 2004b; Sardella & Brauner, 2008a), variation of temperature (Al Amoudi, El-Sayed, & Ghobashy, 1996; Allanson & Bok, 1971; Sardella et al., 2004b; Sardella, Kültz, Cech, & Brauner, 2008), reduced dissolved oxygen levels (Lague, Speers-Roesch, Richards, & Farrell, 2012), and high ammonia concentrations (Suvajdzic, Sardella, & Brauner, 2007). Coupled with use in aquaculture, these remarkable tolerances have made this species a "model invader" (Pérez, Nirchio, Alfonsi, & Muñoz, 2006) and resulted in its listing as one of the most invasive worldwide (Global Invasive Species Database, 2017). More recently, this species has fallen out of favor for use in aquaculture for various reasons (Russell, Thuesen, & Thomson, 2012), yet feral populations have already been established, and in California, it continues to be the primary aquacultural species. In addition to stressor tolerance, there are other aspects of Mozambique tilapia biology that favor

invasiveness; they can exist on a range of food types, and because of their maternal mouthbrooding, young can be transported to new environments over long distances while closed within the oral cavity and protected (Pérez et al., 2006; Russell et al., 2012).

Despite an extreme tolerance to certain variables, low temperature (< 15°C) has proven to be detrimental to tilapia (Cnaani, Gall, & Hulata, 2000; Sardella & Brauner, 2007; Sardella, Cooper, Gonzalez, & Brauner, 2004a). Tilapia are not typically considered eurythermal, yet they do inhabit a modest temperature range of approximately 15–40°C depending, on among other things, environmental salinity (Sardella & Brauner, 2007). Several studies have shown that reduced temperature can depress osmoregulatory function, thus tilapia must make physiological adjustments to survive (Sardella & Brauner, 2007; Sardella et al., 2008). A poor low temperature tolerance may represent why tilapia invasion into more temperate waters has yet to occur, but given physiological robustness of this species, future radiation into such areas should not be dismissed.

Temperature and salinity have been demonstrated to be synergistic stressors for this species. The few studies that have investigated this showed that the factors combined are more stressful than either stress on its own (Al Amoudi et al., 1996; Allanson & Bok, 1971; Sardella & Brauner, 2007; Sardella et al., 2004a). The physiological effects of these

two stressors also appear to be interrelated. Sardella and Brauner (2007) found that a reduction of temperature on its own resulted in the loss of osmotic balance in both freshwater (FW) and seawater (SW). Allanson and Bok (1971) found that a marginal 5 g/l salinity increase was protective from chill coma (11°C) relative to tilapia in FW. In contrast, with respect to thermal preference, Welch, Stauffer, and Morgan (1989) showed that this species selected lower temperatures (2°C cooler) in FW compared with SW.

The purpose of this study was to measure the effects of variable acclimation temperature and salinity on the dynamic thermal tolerance of Mozambique tilapia, as assessed by critical thermal maxima (CT_{Max}) and critical thermal minima (CT_{Min}). To our knowledge, this study was the first to investigate upper and lower dynamic thermal tolerances, and the effect of environmental salinity in this physiologically impressive species. Previously, Stauffer (1986) showed that the "zone of tolerance," defined by the range between incipient upper- and lower-lethal temperatures, was 13–37°C in FW tilapia starting at an acclimation temperature of 25°C (static thermal tolerance). We tested the hypotheses that dynamic thermal tolerance would be affected by acclimation temperature and environmental salinity. To gain insight into how tilapia respond to our thermal challenges, we monitored behavior and quantified ventilation rate throughout.

2 | MATERIALS AND METHODS

2.1 | Fish

Juvenile Mozambique tilapia (*Oreochromis mossambicus*) were provided via a continuous breeding stock at CSU Stanislaus (10.28 ± 1.4 g). Fish were acclimated to FW (3–4 g/l) or two-thirds SW (24 g/l) over a 3-day period and then allowed to acclimate for 2 weeks in five 75 l aquaria outfitted with mechanical, chemical, and biological filtration. After salinity acclimation, temperature was increased/decreased from 28°C to one of five acclimation temperatures; 20, 24 28, 33, or 37°C. Temperature adjustments took place in the 24 and 33°C groups over 24 hr and in the 20 and 37°C groups over 48 hr, and fish were acclimated for another period of 2 weeks once reaching those targets. There were no mortality events resulting from salinity or temperature acclimations, and all tilapia behaviors appeared within normal parameters. Temperature was maintained using submersible aquarium heater (PS$^+$ Aquatics, Flemington NJ, USA) and salinity was manipulated using Instant Ocean Synthetic Sea Salt (Spectrum Brands, Madison WI, USA), which was monitored daily using a portable light refractometer. Tilapia were fed commercially available cichlid food (Cichlid Gold, Hikari Sales, Hayward CA, USA) daily *ad libitum*. Breeding, rearing, and experimentation protocols were approved by California State University Stanislaus Animal Care and Use Committee (IACUC #002-1617).

2.2 | CT_{Max} and CT_{Min}

CT_{Max} and CT_{Min} were measured using the methodology described by Bennett and Beitinger (1997) using loss of equilibrium as the non-lethal endpoint. Tilapia were placed into individual 1-l chambers within a gravity-fed recirculating system, with FW or SW flowing from a header tank containing copper coil connected to a heater/chiller (Forma Scientific 2945 CH/P, Marietta OH, USA). We reliably increased/decreased temperature at a rate of 0.3°C/min using this apparatus. We conducted 20 individual thermal challenges using five fish per challenge (CT_{Max}/CT_{Min} × two salinities × five acclimation temperatures). Temperature was monitored using digital thermometers (Aquarium Brand) in the large chamber as well as each individual exposure chamber. Tilapia were immediately removed from the chamber and temperature recorded when a fish laid down on its side and did not recover following a light touch. During the thermal challenges, opercular beats per minute were counted at increments of ±1°C, and tilapia behaviors were recorded.

2.3 | Analysis

A two-way analysis of variance (ANOVA) was used to test the effects of acclimation temperature, salinity, and T × S interaction on CT_{Max} and CT_{Min}. Significant two-way ANOVA results ($P < 0.05$) were followed by a post-hoc Dunnett's test. Regression lines were also produced for the relationships between CT_{Max}/CT_{Min} and acclimation temperature. Similar to the methods of Bennett and Beitinger (1997), predictive thermal tolerance polygons were constructed using regression trendlines that had been extrapolated to include acclimation temperature extremes successfully-utilized in previous tilapia experiments (high of 37°C from this study, and a low of 15°C from Sardella et al. (2008)). Thermal tolerance polygons were subsequently broken into zones of intrinsic and acquired tolerances (Bennett & Beitinger, 1997; Eme & Bennett, 2009). Zone areas were calculated using the appropriate geometric formulas and expressed as°C^2. All statistical analyses were done using SigmaStat version 4.0 (SYSTAT Software, San Jose CA, USA).

3 | RESULTS

Acclimation temperature, salinity, and temperature/salinity interaction significantly affected acute thermal tolerance limits, as assessed by two-way ANOVA (acclimation temperature ($F = 149.053$, df = 4, $P < 0.001$), salinity ($F = 47.227$, df = 1, $P < 0.001$), and interaction ($F = 14.753$, df = 4, $P < 0.001$)). CT_{Max} ranged from 40.9 to 44.5°C in FW-acclimated tilapia, and from 41.3 to 44.7°C in SW-acclimated tilapia (Table 1). Regression analyses of thermal tolerance limit and acclimation temperature were significant in FW and SW for both CT_{Max} (FW, $P = 0.03$, $r^2 = 0.89$; SW, $P < 0.001$, $r^2 = 0.99$) and CT_{Min} (FW, $P = 0.01$, $r^2 = 0.830$; SW, $P < 0.01$, $r^2 = 0.93$) (Figure 1). Using the regression lines extrapolated to 15°C, we calculated a total thermal tolerance polygon area of 678.9°C^2 for FW-acclimated tilapia, and SW-acclimated tilapia had a total thermal tolerance polygon area of 739.4°C^2 (areas from various zones of tolerance summarized in Table 2).

Throughout the thermal challenges, aquatic surface respiration (ASR; the act of gulping air at the surface and presumably swallowing

TABLE 1 Thermal tolerance limits and scope for FW and SW-Acclimated tilapia

Acclimation Temperature (°C)	FW CT_{Min} ± 95%CI (°C)	FW CT_{Max} ± 95%CI (°C)	FW Thermal Scope (°C)	SW CT_{Min} ± 95%CI (°C)	SW CT_{Max} ± 95%CI (°C)	SW Thermal Scope (°C)
20	8.80 ± 0.32[a#]	40.90 ± 0.37[a]	32.10	6.76 ± 0.37[a#]	41.38 ± 0.07[a]	34.62
24	8.38 ± 0.76[a]	39.78 ± 0.43[b#]	31.40	8.64 ± 0.55[b]	42.36 ± 0.34[b#]	33.72
28	10.35 ± 0.22[b]	42.22 ± 0.36[c#]	31.87	10.74 ± 0.43[c]	42.86 ± 0.51[c#]	32.12
33	12.78 ± 0.33[c]	43.60 ± 0.43[d]	30.82	12.88 ± 0.61[d]	44.04 ± 0.41[d]	31.16
37	16.26 ± 0.67[d#]	44.54 ± 0.13[d]	28.28	12.70 ± 0.40[d#]	44.70 ± 0.35[e]	32.00

Letters within columns represent significant differences in thermal tolerance limit due to acclimation temperature and # denotes a significant effect of salinity within the same acclimation temperature as assessed by two-way ANOVA ($P < 0.001$).
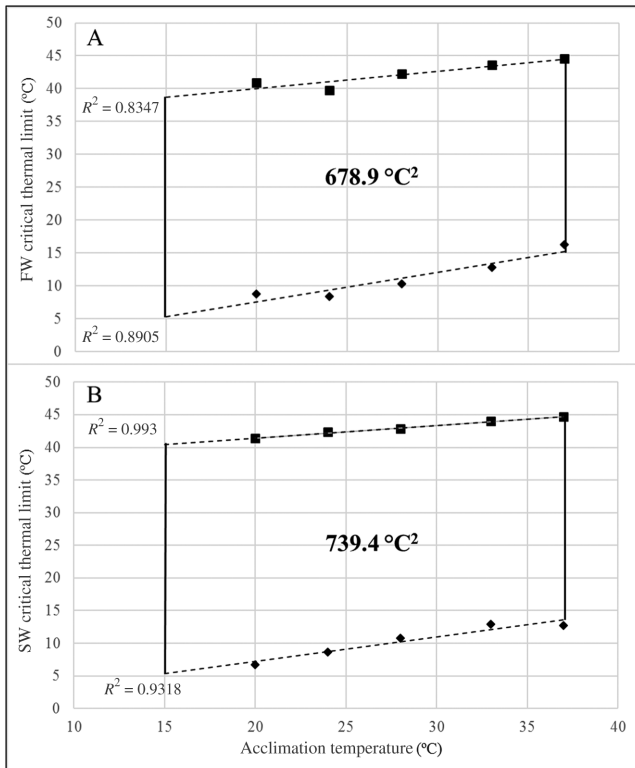

FIGURE 1 Acute thermal tolerance polygons based on predictive regression analyses of CT_{Max} and CT_{Min} with acclimation temperature measured in (A) FW- and (B) SW-acclimated tilapia. Regression lines were extrapolated to the lowest known acclimation temperature utilized in previous work (15°C; Sardella et al., 2008)

it) and ventilation cessation behavior (VCB; the closing of the operculum and mouth for greater than 30 sec) were observed and quantified in both the FW and SW groups (Figure 2). Within the FW-acclimated CT_{Min} tilapia, 80% of individuals exhibited VCB and 16% exhibited ASR, while 88% of FW CT_{Max} fish exhibited ASR and 8% VCB (Figure 2). These behaviors were not as prevalent in SW-acclimated tilapia, with 8% exhibiting ASR and 76% VCB during CT_{Min}, and 56% ASR and no VCB during CT_{Max}.
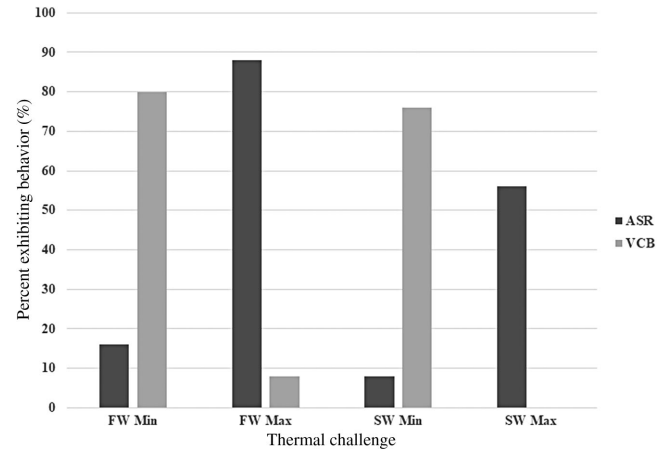

FIGURE 2 Percent of total tilapia thermal challenge exposures in which the tilapia exhibited ventilation cessation behavior (VCB) or aquatic surface respiration (ASR)

4 | DISCUSSION

Tilapia have not been generally regarded as eurythermal fish, likely due to their poor performance in colder temperatures (Cnaani et al., 2000; Sardella & Brauner, 2007; Sardella et al., 2004a; Sardella et al., 2008), yet in this study they exhibited a modest thermal tolerance polygon area of 678.9°C^2 in FW and 739.4°C^2 in SW. The largest documented thermal tolerance polygon area, measured with similar methodology, was seen with sheepshead minnow (*Cyprinodon variegatus*), which exhibited a total area of 1470°C^2 (Bennett & Beitinger, 1997). Relative to the sheepshead minnow, which can tolerate much colder temperatures, which are reflective of its natural habitat (as low as − 1.8°C), tilapia thermal tolerance was not impressive. Overall, tilapia in FW and SW ranked in the middle of species that have been recently investigated using these methods (Table 3). Their thermal tolerance polygons were most similar to rohu (*Labeo rohita*) and catla (*Catla catla*), which are Indian carps (Cyprinidae) that share a similar native habitat to that of the Mozambique tilapia (Das et al., 2004).

TABLE 2 *Calculated* areas (°C^2) *from* thermal tolerance polygons representing *FW- and SW*-Acclimated tilapia

Salinity Acclimation	Upper Acquired Tolerance (°C^2)	Lower Acquired Tolerance (°C^2)	Intrinsic Tolerance (°C^2)	Total Thermal Tolerance (°C^2)
Freshwater	64.0	120.3	494.2	678.9
Seawater	47.3	81.1	611.0	739.4

TABLE 3 Previously published thermal tolerance polygon data for 16 fish species. Bold text represents SW and FW tilapia from this study.

Source	Species	Thermal Tolerance Polygon Area ($°C^2$)
Bennet and Bettinger (1997)	Sheepshead minnow (*Cyprinodon variegatus*)	1470
Ford and Bettinger (2005)	Goldfish (*Carassius auratus*)	1429
Eme and Bennett (2009)	Common goby (*Bathygobius fuscus*)	829
Eme and Bennett (2009)	Squaretail mullet (*Liza viagiensis*)	823
Das,et al. (2004)	Mrigal carp (*Cirrhinus mrigala*)	801.8
Das et al. (2004)	Rohu (*Labeo rohita*)	744.8
	SW *Oreochromis mossambicus*	**739.4**
Das et al. (2004)	Catla (*Catla catla*)	728.8
	FW *Oreochromis mossmbicus*	**678.9**
Dalvi, et al. (2009)	Sun catfish (*Horabagrus brachysoma*)	526.6
Sharma, et al. (2015)	Indian Hill trout (*Barilius bendelisis*)	470.92
Dabruzzi, et al. (2017)	Red lionfish (*Pterois volitans*)	469.6
Eme and Bennett (2009)	Whitetailed humbug (*Dascylus aruanus*)	442
Eme and Bennett (2009)	Nine-banded cardinalfish (*Apogon novemfasciatus*)	408
Dulger et al. (2012)	European sea bass (*Dicentrarchus labrax*)	296.14
Kerfoot (2012)	Pike killifish (*Belonesox belizanus*)	285.9
Campos, et al. (2017)	Green neon tetra (*Paracheirodon simulans*)	235
Campos et al. (2017)	Cardinal tetra (*Paracheirodon axelrodi*)	218

The intolerance of cold temperature in tilapia seems, at the very least, to be linked to osmoregulatory depression and loss of osmotic balance (Sardella et al., 2004a; Sardella et al., 2008). Furthermore, Sardella et al. (2008) showed that this species increased their ionocyte size (SW) and number (FW) as well as total Na^+/K^+-ATPase content when acclimated for 2 weeks to 15°C, yet were still unable to maintain osmotic balance in either salinity. SW-acclimated tilapia thermal limits produced a greater total thermal tolerance polygon area versus FW-acclimated tilapia (Figure 1), due mostly to a higher area of "intrinsic" tolerance (Bennett & Beitinger, 1997). Despite a lesser total thermal tolerance area, FW-acclimated fish produced a greater "acquired" thermal tolerance area (Table 1) indicating that in FW this species may be more capable of invading colder water ways, but will require sufficient acclimation time. The greater intrinsic tolerance of SW tilapia is somewhat puzzling, and certainly worthy of further investigation.

We observed two novel behaviors during our thermal tolerance trials. VCB was described as a closing of the opercula and mouth for a minimum of 30 sec and results in sealing off the gill compartment from the external environment. Aquatic surface respiration (ASR) was also observed, while not a unique behavior to this species or study, it was surprising to observe this behavior in a non-hypoxia-related experiment. To our knowledge, this is the first documentation of VCB in response to environmental stressors, and the first documentation of ASR in response to an environmental stressor other than hypoxia. We conclude that tilapia used these behaviors to extend their thermal tolerance limits.

Both VCB and ASR permit tilapia to limit exposure of the high surface area, high diffusion rate gills to an environmental stressor, which theoretically would also block or reduce exchange. This

potentially limits either direct thermal flux or osmotic movement across the gill epithelium, at least for short durations, but certainly would have detrimental effects with respect to gas exchange (Sardella & Brauner, 2008b). VCB is analogous to breath-hold in air breathing animals, and as such, may result in hypoxemia and hypercapnia within the animal. Tilapia have not previously been shown to exchange gasses across the integument. Furthermore, we have previously measured an oxygen-consumption rate of near zero during VCB behavior (data not presented). However, there have been documented cases of cutaneous respiration by obligate water-breathing species of fishes (Glover, Bucking, & Wood, 2013), so this cannot be totally ruled out without further investigation.

Anecdotally, VCB has also been personally observed in our lab numerous times during exposure to anesthetic, such as MS-222, and even during a low-grade salinity changes. The mouthbrooding strategy used by this species for rearing of young may provide an underlying physiological framework for VCB and hypoxemia, as a mouthbrooding female minimally ventilates and does not open its mouth even to feed (personal observations). Future studies will need to be done to directly document these behaviors and their benefits, as well as which stressors will elicit them.

There were clear behavioral preferences observed in warming versus cooling thermal challenges (Figure 2). The disproportionate use of ASR over VCB in the CT_{Max} groups indicates that increased environmental temperature placed too great a metabolic demand on the fish (Q_{10} effects) making them unable to tolerate the self-imposed hypoxemia that would occur with VCB. Both behaviors limit environmental exposure of the gill epithelium, and we conclude that these protective behaviors may have extended thermal tolerance limits in this study.

On a grander scale, the use of VCB and ASR to extend thermal limits suggests a potential mechanism for the multi-stressor tolerance of this species. This mechanism can only be effective, however, if tilapia can make the physiological adjustments to match energy supply and demand under hypoxemic conditions. Some of the previous work does suggest this may be the case with respect to how they tolerate oxygen-poor water (Lague et al., 2012). Mozambique tilapia are also known as a champions of salinity tolerance, surviving short- and long-term exposures to hypersaline conditions as high as 95 g/l (Sardella & Brauner, 2008a; Sardella et al., 2004b).

In these studies, plasma and muscle osmotic balance was maintained against a remarkably-high gradient without upregulation of any of the well-documented osmo- and ionoregulatory mechanisms (Na^+/K^+-ATPase activity, drinking rate, ionocyte turnover), while oxygen consumption significantly decreased with increasing salinity. Our discovery of VCB during this study provides new insight into how this tilapia potentially defended osmotic balance in those experiments, was able to do so without any osmo- or ionoregulatory adjustments, and also accounts for the reduction of oxygen consumption. We do not suggest that tilapia used VCB throughout the entirety of those experiments, but a modification of this behavior, such as generally lower ventilation rate, to reduce the diffusion/osmotic gradient across the gill should not be discounted. Along with reduced oxygen consumption, tilapia also have been shown to decrease metabolic demand in hypersaline water. Sardella and Brauner, (2008a) showed that liver ATPase activity decreased proportionally with whole animal oxygen consumption as acclimation salinity was increased (range 15–60 g/l). The use of VCB, or ventilation reduction, coupled with a potential metabolic suppression presents an exciting area for future studies, where the multiple stressors that elicit these behaviors can be investigated.

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