Journal of Comparative Physiology B
https://doi.org/10.1007/s00360-025-01633-0

ORIGINAL PAPER

Thermal tolerance plasticity of *Fundulus heteroclitus* is maintained in freshwater and fluctuating temperature conditions

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Received: 14 May 2025 / Accepted: 30 September 2025

Abstract

Understanding how increases in mean temperature and thermal variability impact the physiological flexibility of aquatic species is crucial for predicting the effects of climate change on marine ecosystems. We examined how constant and fluctuating warm temperatures affect thermal tolerance and osmoregulation in Atlantic killifish (*Fundulus heteroclitus*). Killifish were acclimated for ≥4 weeks to control (20 °C), constant (28 °C), and fluctuating (20–28 °C every two days) temperatures in both freshwater (FW) and seawater (SW). Upper thermal tolerance was assessed using critical thermal maximum (CTmax), and plasma osmolality, hematocrit, and glucose levels before and after acute FW→SW and SW→FW transfers. To examine whether long-term acclimation to constant and fluctuating warm temperature is associated with shifts in the steady-state and regulatory responses of gill ionocytes, the gene expression of three branchial ion transporters (*ncc2*, *nkcc1*, and *cftr1*) was measured both before and after an acute salinity transfer. Acclimation to constant and fluctuating warm temperature increased CTmax relative to controls in both FW and SW; however, CTmax was lower in fish acclimated to fluctuating temperature compared to those at constant temperature in FW. Thermal acclimation was linked to an impaired ability to maintain ionic/osmotic homeostasis following an acute transfer from SW→FW, but not from FW→SW. Long-term acclimation to warm temperature in SW was not associated with shifts in either the steady-state or regulatory expression patterns of branchial ion transporters in response to an acute FW transfer. However, upon long-term acclimation to constant and fluctuating warm temperature in FW, we observed an upregulation in the steady-state gene expression of *ncc2*. Our findings highlight the remarkable plasticity of thermal tolerance in *Fundulus heteroclitus* under fluctuating temperature and FW conditions. Additionally, we reveal that thermal acclimation is associated with the upregulation of branchial *ncc2* gene expression, suggesting a compensatory shift in ionocyte function to enhance ion absorption upon long-term acclimation to warm temperature in FW.

Keywords Thermal tolerance · Gill · Ionocyte · Killifish · Osmoregulation · Mummichog

Introduction

Climate change is dramatically affecting marine ecosystems via gradual increases in mean temperatures (Harley et al. 2006; Hoegh-Guldberg and Bruno 2010; Doney et al. 2012). Moreover, climate models predict higher thermal variability associated with frequent and prolonged marine heat waves (Frölicher et al. 2018; Oliver 2018; IPCC 2023). In combination with thermal changes, coastal habitats, including estuaries and salt marshes, are expected to experience more extreme fluctuations in salinity due to increases in net precipitation and freshwater (FW) runoff, as well as rising sea levels and coastal seawater (SW) intrusion (Khojasteh et al. 2021; Costa et al. 2023; Röthig et al. 2023). As temperature and salinity become more dynamic in coastal areas, the physiological challenges faced by native aquatic organisms may shift their distribution, modify their behavior, and push them to their limits of resilience (Doney et al. 2012; Stillman 2019). Therefore, there is an urgent need for a nuanced understanding of how ecologically relevant temperature

Communicated by Graham R. Scott.

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Published online: 17 December 2025

variability affects the physiological flexibility of coastal marine organisms.

Studies examining links between environmental stressors and organismal performance have primarily focused on stable environments (Burggren and Mendez-Sanchez 2023). However, a growing body of evidence suggests that fluctuating and stochastic conditions may influence organismal performance more than gradually changing or constant conditions (Blewett at al. 2022). Many studies incorporating thermal variability show that organisms exhibit distinct responses to constant versus fluctuating temperature conditions (Podrabsky and Somero 2004; Niehaus et al. 2012; Marshall et al. 2021; Ridgeway and Scott 2023); however, this is not a general phenomenon, as some studies report no such differences (Currie et al. 2004; Schwieterman et al. 2022). Additionally, organisms may adopt dissimilar strategies to maintain physiological performance when faced with predictable versus unpredictable temperature shifts (Brown et al. 2024; Nancollas and Todgham 2022). Taken together, these studies indicate that temperature variability and stochasticity impact aquatic organisms in complex ways and emphasize the need to resolve the physiological mechanisms underlying their effects.

As ectothermic organisms whose body temperature is governed by ambient water temperature, fish exhibit profound physiological shifts in response to temperature (Hochachka and Somero 1971; Clarke and Johnston 1999). Specifically, acclimation to increased temperatures leads to physiological adjustments intended to meet higher metabolic demands. Ventilation and cardiac output increase to deliver more oxygen to the gills and tissues, respectively (Chen et al. 2018). Additionally, morphological remodeling of the branchial epithelium, which involves the regression of interlamellar cell masses, increases the surface area for gas exchange (Nilsson 2007; Gilmour and Perry 2018; Wood and Eom 2021; Gilmour and Turko 2024). While gill remodeling in response to increased temperature enhances O_2 uptake, it also has the potential to exacerbate branchial ion and water fluxes, a trade-off coined the 'osmorespiratory compromise' (Randall et al. 1972; Gilmour and Perry 2018; Wood and Eom 2021). This compromise arises because efficient gas exchange requires a large branchial functional surface area and a short diffusion distance. In contrast, the opposite conditions are expected to decrease the energetic cost of ion/osmoregulation by reducing passive ion and water fluxes. Therefore, the physiological adjustments required for acclimation to increased temperature may present a challenge to ionic/osmotic homeostasis unless fish can employ compensatory strategies.

The challenges to ionic/osmotic homeostasis differ for fish inhabiting FW versus SW environments. In SW, fish are hyposmotic to their environment and must combat diffusive water loss and passive ion gain. This is accomplished through the active secretion of Na^+ and Cl^- by 'SW-type' ionocytes in the branchial epithelium (Hiroi and McCormick 2012; Takei et al. 2014). The strategy for branchial salt secretion in fishes resembles that of other vertebrate secretory epithelia and involves three ion transport proteins: basolateral Na^+/K^+-ATPase (Nka) and $Na^+/K^+/2Cl^-$ cotransporter 1 (Nkcc1), and the apical Cl^- channel, cystic fibrosis transmembrane conductance regulator 1 (Cftr1). Nka generates the electrical and chemical gradients that drive Na^+ and Cl^- into the cell via Nkcc1. Then, Na^+ and Cl^- exit the gill through a paracellular pathway and Cftr1, respectively. In contrast to marine/SW-acclimated fish, FW fish are hyperosmotic to their environment. They must therefore counteract passive water gain and ion loss by producing dilute urine and absorbing ions across the branchial epithelium. Ion absorption involves the coordinated activities of several transport proteins located in 'FW-type' ionocytes, including Na^+/Cl^- cotransporter 2 (Ncc2); however, ionocyte-based strategies for Na^+ and Cl^- uptake vary among species (Hiroi and McCormick 2012; Kovac and Goss 2024). The ability to maintain ionic/osmotic homeostasis in both SW and FW environments also depends on the permeability characteristics of the branchial epithelium (Chasiotis et al. 2012). In SW, the gill is 'leaky' to facilitate the paracellular secretion of Na^+, whereas in FW, the gill is 'tight' to limit passive ion loss.

Recent studies in euryhaline models examined the impacts of warm temperature on branchial ionocytes and their ion transporters. In the European seabass (*Dicentrarchus labrax*), exposure to warm temperature and low salinity resulted in a reduction of ionocyte numbers (Masroor et al. 2019) and a decrease in *nka* and *nkcc1* expression (Islam et al. 2020). In Atlantic salmon (*Salmo salar*), exposure to warm temperature was associated with poor ion regulation in SW, as well as reduced branchial Nka activity and Nkcc1 abundance (Vargas-Chacoff et al. 2018). In another study, warm temperature was shown to modulate the osmoregulatory capacity and branchial Nka activity of gilthead sea bream (*Sparus aurata* L.) (Vargas-Chacoff et al. 2020). Together, these studies indicate that acclimation to warm temperature may lead to shifts in the density and gene expression patterns of branchial ionocytes. However, much remains unknown regarding the effects of constant versus fluctuating warm temperature on whole-animal osmoregulation and the gene expression patterns of ion transporters within 'FW-type' and 'SW-type' ionocytes.

The current study employs the euryhaline fish model, Atlantic killifish (*Fundulus heteroclitus*), because they play an important role in the ecology of coastal marine ecosystems and exhibit extreme tolerances to temperature and salinity (Griffith 1974; Kneib 1986; Burnett et al. 2007). It

is well-known that killifish exhibit substantial thermal tolerance plasticity, with warm acclimation leading to gains in upper thermal tolerance as measured by critical thermal maximum (CTmax) methodologies (Fangue et al. 2006; Healy and Schulte 2012). However, these studies were conducted with animals held at 20 ppt under constant temperature conditions. Recent work in this species showed that killifish improve their hypoxia tolerance after acclimation to both constant and fluctuating warm temperature conditions when held at 4 ppt. This demonstrates the plasticity of hypoxia tolerance in response to warm temperatures when killifish are acclimated to low salinity (Ridgeway and Scott 2023). However, little is known about thermal tolerance plasticity of killifish under fluctuating temperature and FW conditions. In addition, the ionoregulatory mechanisms employed by killifish in FW are distinct from those of other euryhaline species (Takei et al. 2014) (i.e., salmon) for which the effects of warm temperature acclimation have been studied (Vargas-Chacoff et al. 2018).

The objectives of this study were to compare the effects of constant and fluctuating warm temperature on thermal tolerance, osmoregulation, and branchial ionocytes in both FW and SW. We predicted that a conflict between respiration and osmoregulation would constrain thermal tolerance plasticity in FW, or, if that were not the case, then compensatory shifts in branchial ionoctye function would be necessary. In two separate trials, killifish were subjected to three temperature treatments: control (20 °C), constant warm (28 °C), and fluctuating warm (20–28 °C, changing every two days). We examined how these treatments affected the upper thermal tolerance and hydromineral balance of fish acclimated to either SW (30 ppt) or FW (0 ppt) and acutely transferred to the opposite salinity. We also characterized the branchial expression patterns of *ncc2*, *nkcc1*, and *cftr1* to examine whether thermal acclimation leads to shifts in the steady-state expression and/or regulatory response of ionocytes to an acute salinity transfer. Specifically, expression of *ncc2* was used to examine the impact of warm temperature on 'FW-type' ionocytes, whereas *nkcc1* and *cftr1* were used to examine impacts on 'SW-type' ionocytes.

Materials and methods

Animals and rearing conditions

Atlantic killifish (≥4.0 g) from the northern subspecies (*Fundulus heteroclitus macrolepidotus*) were field-collected by Aquatic Research Organisms, Inc. in Hampton, NH (Latitude: 42° 55′ 20.9″ N; Longitude: 70° 51′ 08.9″ W) and housed at Western Connecticut State University (Danbury, CT). Killifish were maintained in 75-L rectangular glass aquaria containing FW (0 ppt) or SW (30 or 35 ppt). Artificial SW was prepared using dechlorinated Danbury City tap water and Instant Ocean Aquarium Sea Salt (Blacksburg, VA), and salinity was determined using a YSI Pro salinity meter. Water in all aquaria was continuously recirculated, particle and charcoal filtered, aerated, and maintained at 20 °C. Daily measurements of temperature, salinity, pH, ammonia, nitrites, and nitrates were recorded, and water changes were performed as necessary. Fish were exposed to a 12-h light: dark cycle and fed to satiation once daily with TetraMin® fish flakes. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Western Connecticut State University.

Temperature acclimations

To examine the effects of warm temperature acclimation on the thermal tolerance and osmoregulation of killifish, two separate studies (2022 and 2023) were conducted using different cohorts of field-collected fish (Figs. 1 and 2). In both studies, fish were held under the laboratory conditions described above and then assigned to one of three temperature treatments: control (20 °C), constant warm (28 °C), and fluctuating warm (20–28 °C). Water temperatures were chosen to represent ecologically relevant conditions of coastal marine waters in the Northeastern United States during late summer and early fall. For the fluctuating warm treatment, temperature was changed from 20 to 28 °C every two days to simulate repeated, successive heatwaves. In 2022, temperature acclimations were conducted in SW (30 ppt), while in 2023, they were conducted in FW (0 ppt). In both studies, fish were acclimated to the three temperature treatments for ≥4 weeks. Water temperatures were recorded hourly using HOBO data loggers (Bourne, MA).

Thermal tolerance

Thermal tolerance was measured using the critical thermal maximum methodology outlined by Fangue et al. (2006). This methodology defines the critical thermal maximum (CTmax) as the upper temperature at which loss of equilibrium is observed. CTmax trials were conducted with a subset of fish from each temperature treatment. For the fluctuating treatment group, CTmax trials were performed with fish removed from the acclimation tank at both 20 °C and 28 °C. Trials were performed in a test chamber consisting of a rectangular glass water bath containing five individual 1-L plastic beakers. One fish was placed in each beaker containing water set to the appropriate acclimation salinity and a temperature of 24 °C, representing the midpoint of the temperature treatments. The water bath was heated at a target rate of 0.3 °C min^{-1} using a copper immersion coil

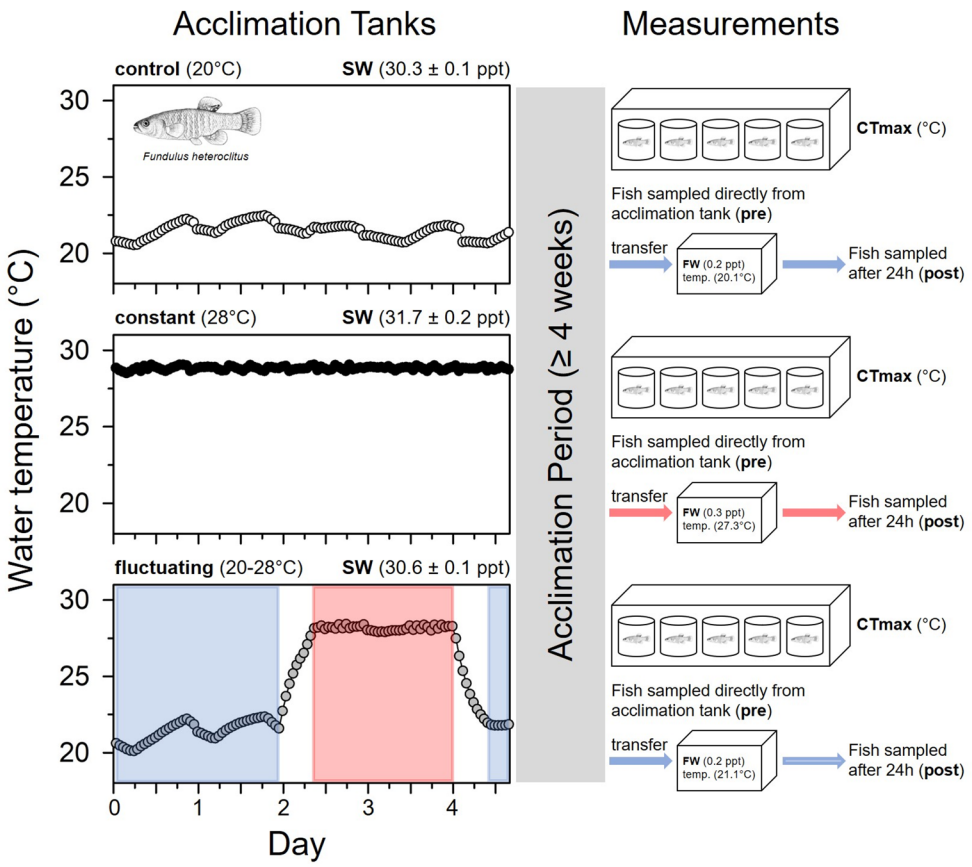

Fig. 1 Experimental design of the 2022 study. Killifish were first acclimated to control (20 °C), constant (28 °C), or fluctuating (20–28 °C) temperature in seawater (SW, 30 ppt). Graphs show representative temperature data for the three acclimation tanks recorded hourly with HOBO data loggers. For the fluctuating treatment, water temperature was changed from 20 to 28 °C (or vice versa) every two days, and fish were exposed to ~9 pulses of warm temperature during the acclimation period. The temperature ramp-up and ramp-down times were ~12 h. Following ≥4 weeks of acclimation, upper thermal tolerance was measured in a subset of fish removed from each acclimation tank using critical thermal maximum (CTmax) methodology. In vivo whole-animal measurements and in vitro tissue measurements were made in subsets of fish either sampled directly from their acclimation tank (pre) or after transfer to freshwater (FW, 0 ppt) for 24 h (post). For the fluctuating treatment only, CTmax was measured in fish removed from the acclimation tank at both 20 °C and 28 °C, and FW transfers were conducted at 20 °C. Actual temperatures and salinities recorded during the acclimation period and acute salinity transfers are shown

connected to an Isotemp 36TD benchtop unit. The water bath was circulated with submersible pumps to ensure complete mixing. Individual beakers were continuously aerated to maintain oxygen concentrations at saturation, and temperatures were monitored with digital thermometers. During each trial, individual fish were continuously monitored, and a loss of equilibrium, defined as failure to maintain dorsal-ventral orientation for more than 10 s, was used as the CTmax endpoint. All individuals recording CTmax were blind to the treatment group. When equilibrium was lost, fish were immediately removed from the test beakers and placed into an anesthesia bath (MS-222, 500 mg l^{-1}, Syndel, Ferndale, WA) prior to sampling.

Freshwater and seawater challenge tests

To examine the effect of warm temperature acclimation on acute osmoregulatory ability, a subset of fish from each temperature treatment was transferred directly from their acclimation tank to a glass aquarium containing water of the opposite salinity (30 ppt → 0 ppt in 2022 and 0 ppt → 35 ppt in 2023); water temperature was consistent with acclimation treatment. Fish were fasted throughout the salinity challenge test and sampled after 24 h. Plasma osmolality served as an index of acute osmoregulatory ability.

Sampling of fish tissues and blood analysis

Upon sampling, all fish were anesthetized with MS-222, length-measured, and weighed. Blood samples were collected from the severed caudal vein using heparinized

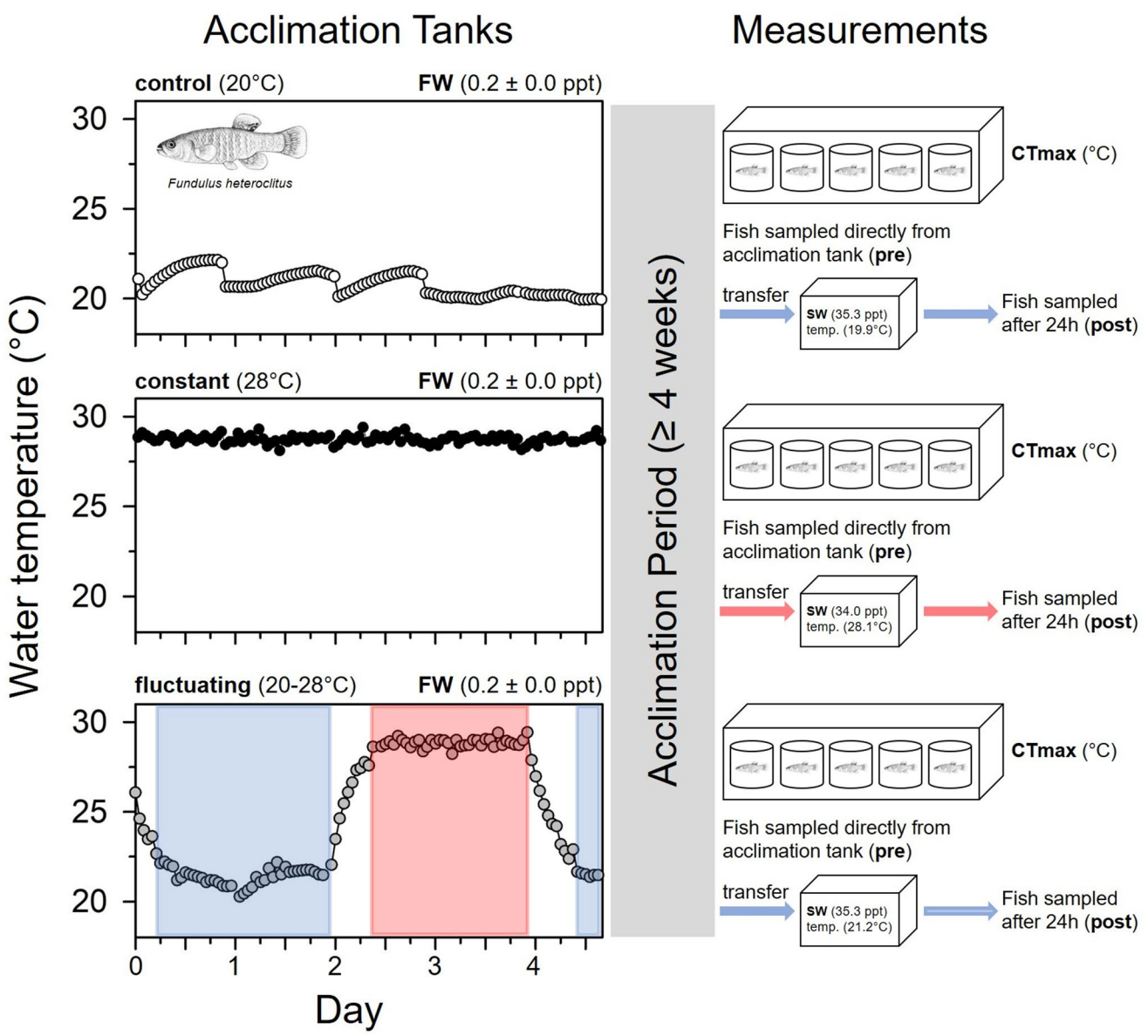

Fig. 2 Experimental design of the 2023 study. Killifish were first acclimated to control (20 °C), constant (28 °C), or fluctuating (20–28 °C) temperature in freshwater (FW, 0 ppt). Graphs show representative temperature data for the three acclimation tanks recorded hourly with HOBO data loggers. For the fluctuating treatment, water temperature was changed from 20 to 28 °C (or vice versa) every two days, and fish were exposed to ~10 pulses of warm temperature during the acclimation period. The temperature ramp-up and ramp-down times were ~12 h. Following≥4 weeks of acclimation, upper thermal tolerance was measured in a subset of fish removed from each acclimation tank using critical thermal maximum (CTmax) methodology. In vivo whole-animal measurements and in vitro tissue measurements were made in subsets of fish either sampled directly from their acclimation tank (pre) or after transfer to seawater (SW, 35 ppt) for 24 h (post). For the fluctuating treatment only, CTmax was measured in fish removed from the acclimation tank at both 20 °C and 28 °C, and SW transfers were conducted at 20 °C. Actual temperatures and salinities recorded during the acclimation period and acute salinity transfers are shown

microcapillary tubes. Blood samples were centrifuged for 5 min at room temperature in a benchtop microhematocrit centrifuge (12,700 x g), and hematocrit (%) was measured using a microcapillary reader. Plasma was stored at −80 °C for subsequent analysis. Gill arches from the left side were removed, blotted, immersed in RNAlater Stabilization Solution (Invitrogen), and stored at −80 °C. Plasma osmolality was measured using a vapor pressure osmometer (Wescor 5600, Logan, UT). Plasma glucose was measured by enzymatic coupling with hexokinase and glucose-6-phosphate dehydrogenase (Millipore Sigma, Burlington, MA) using a BioTek microplate reader.

RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

After removal from RNAlater Stabilization Solution, gill filaments were homogenized in TRI Reagent (Molecular Research Center, Cincinnati, OH) to extract total RNA following the manufacturer's protocol. RNA concentration and purity ($1.9 < A260/A280 < 2.2$) were assessed by spectrophotometric absorbance (NanoDrop One, ThermoFisher Scientific, Waltham, MA). First strand cDNA was synthesized by reverse transcribing 100 ng of total RNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Relative levels of mRNA were determined by qRT-PCR using the StepOnePlus real-time PCR system (Life Technologies). We employed previously described primer sets for all target and reference genes aside from *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) (Table 1). Primers for *gapdh* (XM_012870348.3) were designed using NCBI Primer-BLAST to span a predicted exon-exon junction and to amplify a product of 149 base pairs. Non-specific product amplification and primer-dimer formation were assessed by melt curve analysis and gel electrophoresis. Only samples with melt curves containing a single peak for each primer set were used to determine gene expression ratios. The *gapdh* primers exhibited an efficiency of 92%. qRT-PCR reactions were performed in a 15 µl volume containing 2X Power SYBR Green PCR Master Mix (Life Technologies), 200 nmol l^{-1} of each primer, nuclease free water, and 1 µl cDNA template. The following cycling parameters were employed: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. After confirming that levels did not vary across the treatments in a given experiment, either *elongation factor 1α* (*ef1α*) or *gapdh* levels were used to normalize target genes. Reference and target genes were calculated by the relative quantification method with PCR efficiency correction (Pfaffl 2001). Standard curves were prepared from serial dilutions of control gill cDNA and included on each plate to calculate the PCR efficiencies for target and reference gene assays. Negative reverse transcriptase control samples were used to screen for genomic DNA contamination. Relative gene expression ratios between groups are reported as a fold-change from controls.

Table 1 Specific primer sequences for quantitative real-time PCR

Gene	Primer Sequence (5′−3′)	Reference/Acc. No.
cftr1	F: AATCGAGCAGTTCCCAGACAAG R: AGCTGTTTGTGCCCATTGC	Scott et al. 2005
ef1α	F: GGGAAAGGGCTCCTTCAAGT R: ACGCTCGGCCTTCAGCTT	Scott et al. 2005
gapdh	F: CATGAAGGGTGTCCTGGGAT R: CGTACTCGTTGTCGTACCATGAAA	XM_012870348.3
ncc2	F: AGTCACATCCTGACCGGAAAC R: TCACAGGACTGAGACTGGAT	Breves et al. 2020
nkcc1	F: CCCGCAGCCACTGGTATT R: GCCATCTGTGGGTCAGCAA	Scott et al. 2004

Statistics

One- and two-way ANOVA were used to test for the main effects of treatment (control, constant, fluctuating) and salinity (FW, SW) depending on the experimental design. Shapiro-Wilk and Brown-Forsythe tests were used to test for the assumptions of normality and equal variance, respectively. When necessary, data were rank-transformed to meet the assumptions of ANOVA. When significant main effects were observed, Tukey *post hoc* tests were used for multiple comparisons. Statistical analyses were performed using Sigma Plot (version 13). The significance level for all tests was set at $p < 0.05$.

Results

Effects of constant and fluctuating warm temperature on thermal tolerance

Figure 3A shows the effect of constant and fluctuating warm temperature on upper thermal tolerance of fish acclimated to SW (30 ppt). Mean ± s.e.m. CTmax of fish in the control treatment was 39.6 ± 0.3 °C, consistent with previously reported values for this species at similar salinities and temperatures (Fangue et al. 2006; Healy and Schulte 2012). Acclimation temperature strongly affected thermal tolerance as indicated by a significant main effect of treatment on CTmax ($p < 0.001$, Fig. 3A). Warm acclimation led to substantial gains in thermal tolerance; CTmax was elevated in both the constant (+2.6 °C) and fluctuating (+1.8 °C) temperature groups relative to controls (Fig. 3A). Mean CTmax was slightly lower in the fluctuating temperature group compared to the constant group; however, this difference was not statistically significant ($p = 0.127$, Fig. 3A). For the fluctuating group, CTmax trials were performed with fish removed from the acclimation tank at both 20 °C and 28 °C. When visualized separately, we observed that CTmax was slightly lower in fish removed at 20 °C as compared to fish at 28 °C; however, this difference was not statistically significant (t-test, $p = 0.126$, Fig. 3A).

Figure 3B shows the effect of constant and fluctuating warm temperature on upper thermal tolerance of fish acclimated to FW (0 ppt). Mean CTmax of fish in the control treatment was 37.1 ± 0.3 °C, which represents a lower value

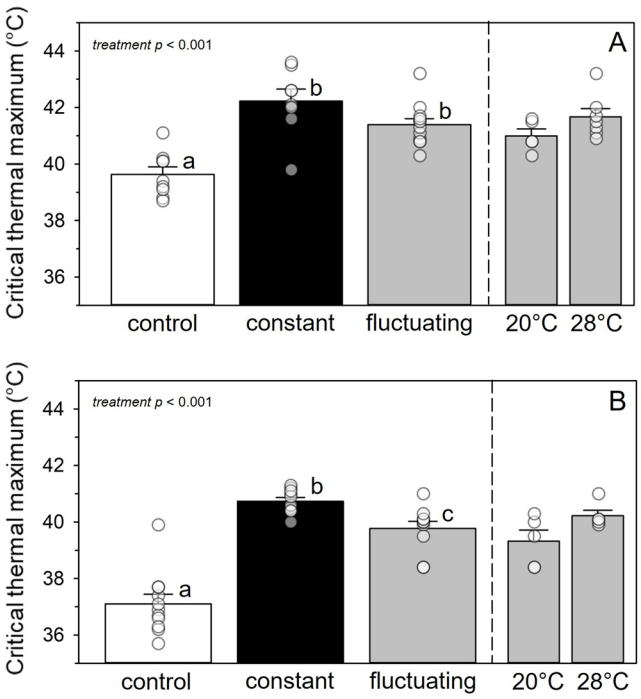

Fig. 3 Critical thermal maximum (CTmax) of killifish acclimated to control (20 °C), constant (28 °C), and fluctuating (20–28 °C) temperature in seawater (A) and freshwater (B). Bars represent mean ± s.e.m. (n = 5–12) and circles represent individual values. Dissimilar letters indicate a significant difference in pairwise comparisons between temperature acclimation groups. Bars to the right of the dashed line show the CTmax of fish from the fluctuating temperature group separated by the acclimation tank temperature (20or 28 °C) on the day of CTmax trials

than that observed for the control group in the SW study. Acclimation temperature strongly affected thermal tolerance as indicated by a significant main effect of treatment on CTmax (p < 0.001, Fig. 3B). Warm acclimation led to substantial gains in thermal tolerance; CTmax was elevated in both the constant (+3.8 °C) and fluctuating (+3.2 °C) temperature groups relative to controls (Fig. 3B). Mean CTmax was significantly lower (−1.0 °C) in the fluctuating temperature group compared to the constant group (Fig. 3B). Like above, CTmax trials were performed with fish removed from the fluctuating acclimation tank at both 20 °C and 28 °C. When visualized separately, we observed that CTmax was slightly lower in fish removed at 20 °C as compared to fish at 28 °C; however, this difference did not reach statistical significance (t-test, p = 0.077, Fig. 3B).

Effect of warm temperature on osmoregulation in freshwater and seawater

To examine the effect of warm temperature on steady-state and acute osmoregulation, plasma osmolality was measured in killifish acclimated to the three temperature treatments and sampled both before (pre) and after (post) salinity transfer. The effect of acclimation to constant and fluctuating warm temperature on steady-state osmoregulation in SW, and acute osmoregulation in response to transfer from SW→FW is shown in Fig. 4A. We observed a significant main effect of salinity (p < 0.001) and a significant treatment*salinity interaction (p = 0.028) on plasma osmolality (Fig. 4A). Warm acclimation resulted in significant reductions in plasma osmolality in response to transfer from SW→FW in both the constant and fluctuating temperature groups, but not in controls (Fig. 4A). The effect of acclimation to constant and fluctuating warm temperature on steady-state osmoregulation in FW, and acute osmoregulation in response to transfer from FW→SW is shown in Fig. 4B. We observed a significant main effect of salinity only on plasma osmolality (p < 0.001), with levels elevated in response to FW→SW transfer in all treatment groups (Fig. 4B). While acclimation to constant and fluctuating warm temperature did not appear to impact steady-state osmoregulation in either acclimation salinity (Fig. 4A, B), acclimation to warm temperature led to a reduction in acute osmoregulatory ability upon transfer of killifish from SW→FW (Fig. 4A).

Effect of warm temperature on plasma indices in freshwater and seawater

We examined the effect of warm temperature on plasma indices, hematocrit and plasma glucose, in killifish acclimated to the three temperature treatments and sampled both before and after salinity transfer. We observed that hematocrit was not significantly affected by treatment, salinity, or their interaction when acclimated to the three temperature treatments in SW (Fig. 4C); however, there were significant main effects of treatment (p = 0.003) and salinity (p < 0.001) on plasma glucose (Fig. 4E). Mean plasma glucose levels increased following SW→FW transfer in both the control and constant groups; however, this effect was not observed in the fluctuating group (Fig. 4E). When killifish were acclimated to the three temperature treatments in FW, we observed a significant treatment*salinity interaction on hematocrit (p = 0.003, Fig. 4D). Hematocrit was decreased in response to FW→SW transfer in both the constant and fluctuating groups; however, this effect was not observed in the control group (Fig. 4D). We observed a significant main effect of treatment on plasma glucose levels (p = 0.020, Fig. 4F). Plasma glucose levels were elevated in the constant group relative to the control group when fish were sampled after 24 h in SW (Fig. 4F). Together, our data indicate that acclimation to warm temperature alone does not have large impacts on indices of whole-animal physiology such as hematocrit and plasma glucose. This aligns with the temperature treatments employed in our study

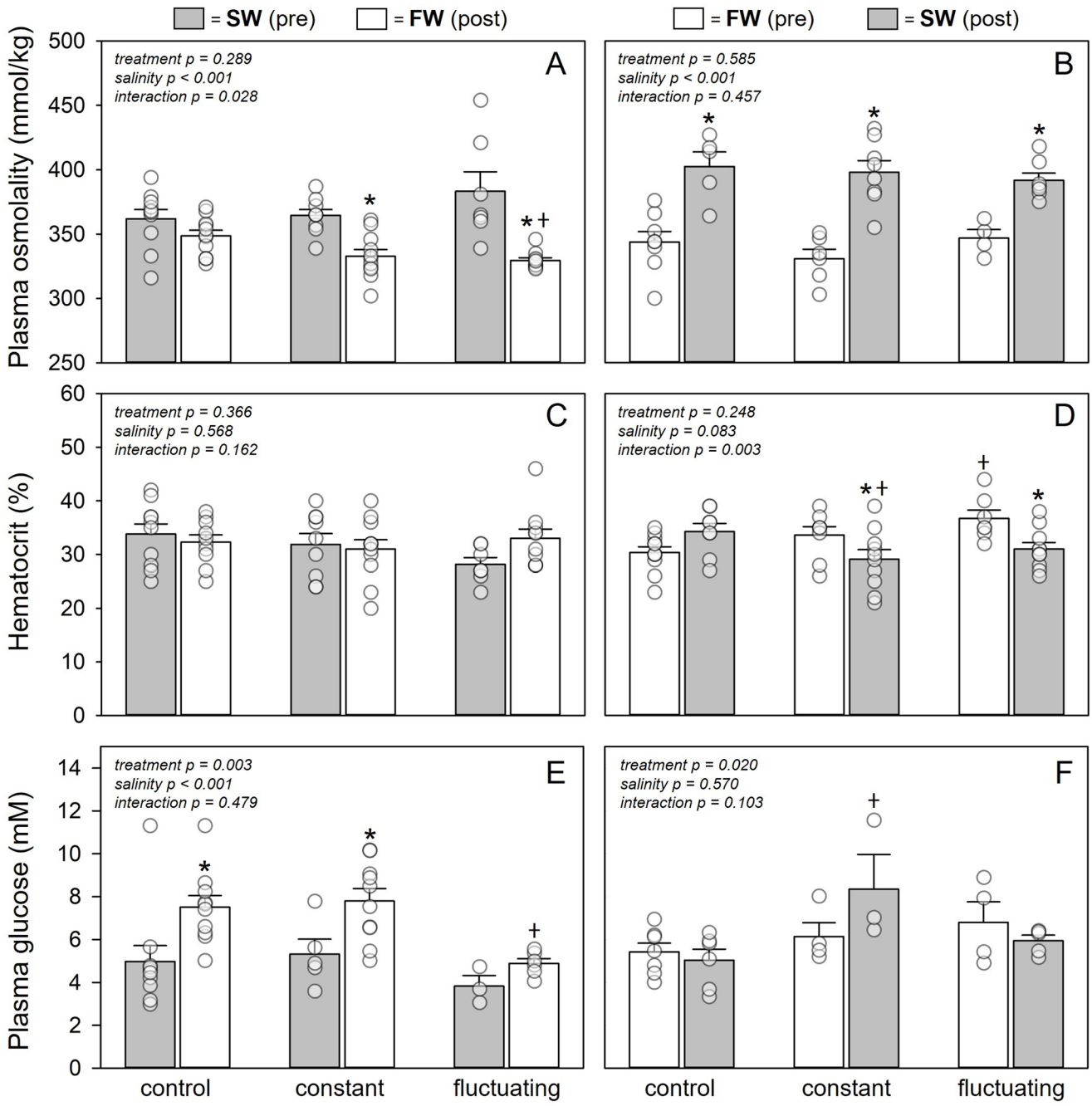

Fig. 4 Plasma osmolality (**A**, **B**), hematocrit (**C**, **D**), and glucose (**E**, **F**) of killifish acclimated to control (20 °C), constant (28 °C), and fluctuating (20–28 °C) temperature. Fish in each temperature treatment were sampled directly from their acclimation tank, and 24 h after transfer from seawater to freshwater (SW→FW, left panels) or freshwater to seawater (FW→SW, right panels). Bars represent mean ± s.e.m. (n = 3–11) and circles represent individual values. An asterisk indicates significant differences between salinities within a treatment. A cross indicates significant differences from the control within a salinity

being well within the range of temperatures tolerated by killifish (Fangue et al. 2009). In contrast, an acute transfer from FW→SW is associated with reductions in hematocrit in warm-acclimated fish, while transfer from SW→FW resulted in increased plasma glucose levels in both the control and constant groups.

Effect of warm temperature on branchial ion transporter gene expression in freshwater and seawater

To examine the effects of warm temperature acclimation on branchial ionocytes, we measured the gene expression of *ncc2*, *nkcc1*, and *cftr1* in killifish acclimated to the three temperature treatments and sampled both before (pre) and after (post) salinity transfer. Figure 5 shows the effect of warm temperature on the gene expression of branchial ion transporters in fish acclimated to SW and following an acute transfer from SW→FW. For all three ion transporters, we observed a significant main effect of salinity ($p<0.001$, Fig. 5A-C), with the dynamics of each gene consistent with previous descriptions of killifish undergoing FW acclimation (Scott et al. 2004; Breves et al. 2020). *ncc2* expression increased in response to SW→FW transfer across all treatment groups (Fig. 5A), whereas the expression levels of *nkcc1* (Fig. 5B) and *cftr1* (Fig. 5C) were reduced. Since no significant treatment or interaction effects were observed for the three examined genes, acclimation to warm temperature in SW is not associated with shifts in branchial ion transporter gene expression under steady-state conditions or following an acute SW→FW transfer.

Figure 6 shows the effect of warm temperature on the gene expression of branchial ion transporters in fish acclimated to FW and subsequently transferred to SW. We observed a significant main effect of salinity on *ncc2* expression ($p<0.001$) and a significant treatment*salinity interaction ($p=0.002$) (Fig. 6A). *ncc2* expression was decreased in all treatment groups in response to FW→SW transfer; however, this decrease did not reach statistical significance under control conditions ($p=0.127$, Fig. 6A). We also observed that *ncc2* expression was elevated in the constant and fluctuating groups relative to controls when fish were sampled in FW prior to SW transfer (pre). *ncc2* expression was also reduced in the fluctuating group relative to controls after 24 h in SW (Fig. 6A). For *nkcc1* expression, we observed a significant main effect of salinity only ($p<0.001$); *nkcc1* expression was elevated in response to FW→SW transfer across all treatment groups (Fig. 6B). We observed a significant main effect of salinity on *cftr1* expression ($p<0.001$; Fig. 6C). Like *nkcc1*, *cftr1* expression was elevated in response to FW→SW transfer in all treatment groups (Fig. 6C). Significant salinity and interaction effects were observed for *ncc2*; therefore, acclimation to warm temperature in FW is associated with shifts in branchial ion transporter gene expression under steady-state conditions and following an acute transfer from FW→SW.

Discussion

Elevated thermal tolerance is maintained under fluctuating temperature conditions

Previous studies show that warm temperature acclimation substantially increases upper thermal tolerance in killifish (Fangue et al. 2006; Healy and Schulte 2012; Drown et al. 2021). However, these studies focused on examining the effects of warm temperature under constant laboratory conditions. Given that killifish experience significant seasonal and daily fluctuations in water temperature in their native habitat (Schulte 2007), our first objective was to determine whether they exhibit elevated thermal tolerance when exposed to fluctuating periods of cool (20 °C) and warm (28 °C) temperature. Our results show that CTmax in the fluctuating temperature groups is significantly elevated relative to controls (Fig. 3), with similar responses in FW and SW. This result is consistent with previous work in killifish showing that gaining thermal tolerance in response to warm temperature occurs faster than losing tolerance in response to cool temperature (Healy and Schulte 2012). These authors reported that increases in CTmax were detectable 1 day after transfer from 15 °C to 25 °C. In contrast, upon transferring fish from 15 °C to 5 °C, CTmax had still not decreased to values expected for fully 5 °C acclimated fish after 3 weeks (Healy and Schulte 2012). We also observed that the CTmax of fish in the fluctuating groups was lower than that of fish maintained at constant warm temperature, particularly in FW (Fig. 3). Therefore, we suggest that under fluctuating conditions, killifish maintain the thermal tolerance gained during warm temperature (28 °C) periods; however, some loss of thermal tolerance may occur within 2 days after exposure to cool temperature (20 °C).

Our results agree with previous findings on how constant and fluctuating warm temperatures affect hypoxia tolerance in killifish (Ridgeway and Scott 2023), which is logical because both thermal and hypoxia tolerance may depend on the ability to deliver O_2 to tissues (Healy and Schulte 2012; McBryan et al. 2016). Ridgeway and Scott (2023) determined that acclimation to a constant warm temperature increased hypoxia tolerance at that specific temperature but resulted in poor hypoxia tolerance at other temperatures, indicating that acclimation to a constant temperature leads to thermal specialization. For fish experiencing fluctuating temperatures, hypoxia tolerance was intermediate or nearly

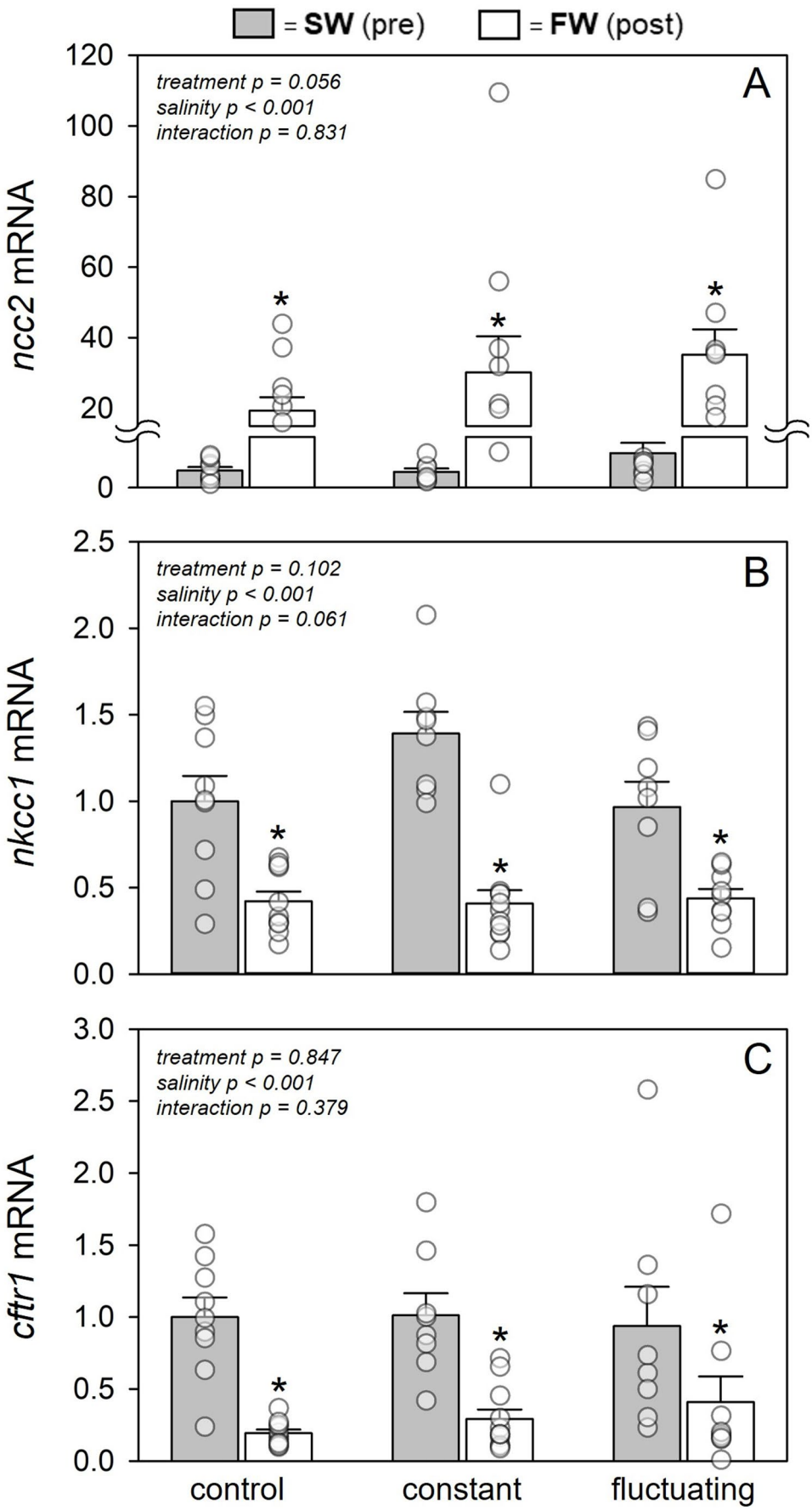

Fig. 5 Branchial gene expression of *ncc2* (**A**), *nkcc1* (**B**), and *cftr1* (**C**) in killifish acclimated to control (20 °C), constant (28 °C), and fluctuating (20–28 °C) temperature. Fish in each temperature treatment were sampled directly from their acclimation tank, and 24 h after transfer from seawater to freshwater (SW→FW). mRNA levels are presented as a fold-change from the SW control group. Bars represent mean ± s.e.m. (n = 8–11) and circles represent individual values. An asterisk indicates significant differences between salinities within a treatment

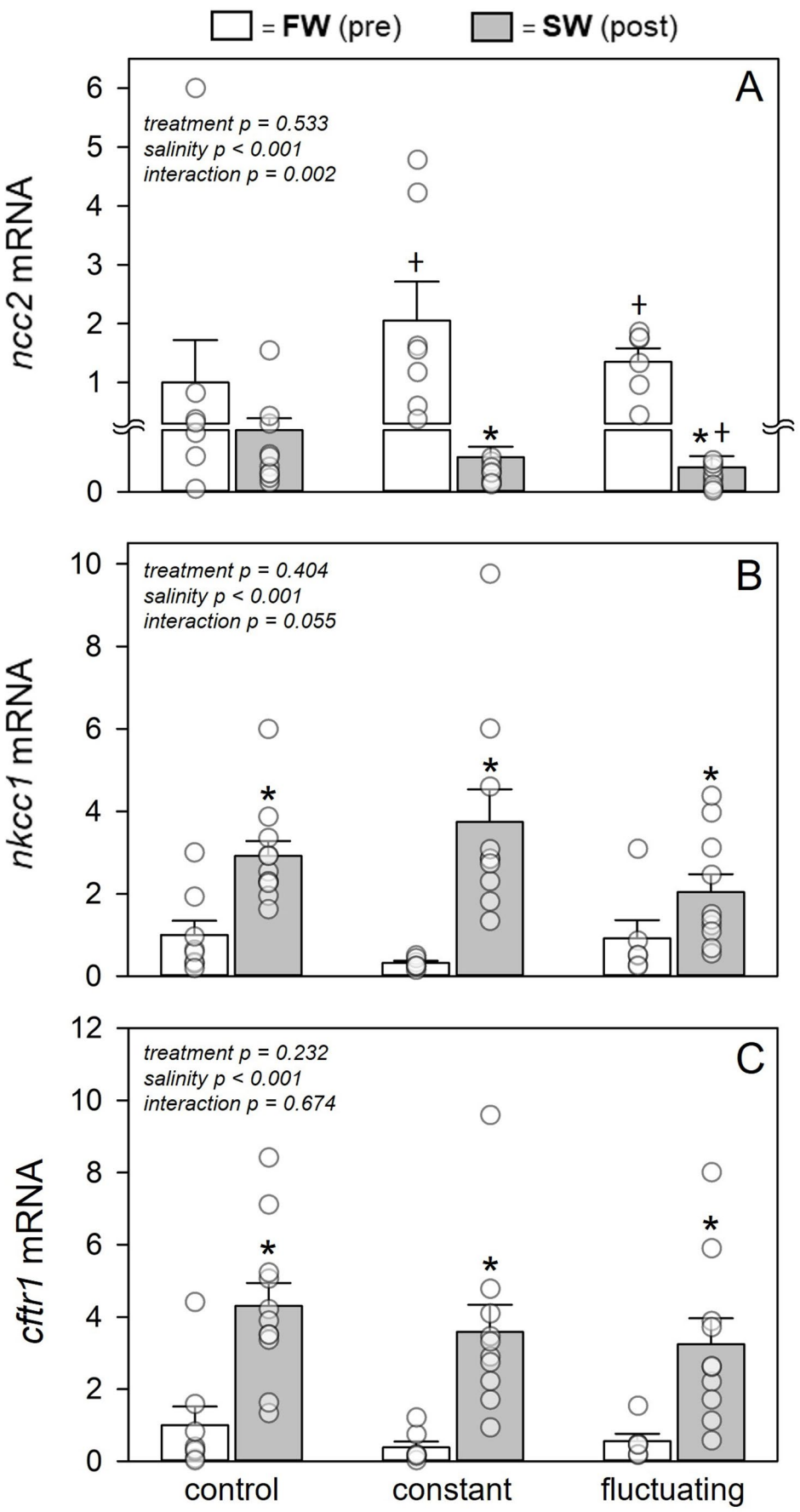

Fig. 6 Branchial gene expression of *ncc2* (**A**), *nkcc1* (**B**), and *cftr1* (**C**) in killifish acclimated to control (20 °C), constant (28 °C), and fluctuating (20–28 °C) temperature. Fish in each temperature treatment were sampled directly from their acclimation tank, and 24 h after transfer from freshwater to seawater (FW→SW). mRNA levels are presented as a fold-change from the FW control group. Bars represent mean ± s.e.m. (n = 6–11) and circles represent individual values. An asterisk indicates significant differences between salinities within a treatment. A cross indicates significant differences from the control within a salinity

as high as that of fish under constant temperature conditions; however, hypoxia tolerance was better maintained across a broader range of cool temperatures, indicating a more generalist strategy. Moreover, Ridgeway and Scott (2023) associated these differences in hypoxia tolerance with variations in blood hemoglobin content, hematocrit, and hemoglobin-O_2 affinity. Taken together, the results of our study support the conclusion that killifish maintain plasticity of thermal tolerance under fluctuating temperature conditions and that this physiological capacity is similar in both FW and SW environments. Nonetheless, we observed that killifish failed to maintain the highest level of thermal tolerance under fluctuating temperature conditions; therefore, our data add to the growing evidence that thermal history influences physiological performance traits such as thermal and hypoxia tolerance.

Thermal tolerance plasticity is maintained in freshwater

Previous studies on thermal tolerance plasticity in killifish were conducted with individuals held at ~20 ppt, the preferred salinity for this species (Garside and Jordan 1968; Fritz and Garside 1974; Bucking et al. 2012; Marshall et al. 2016). However, killifish move freely between salinities in their native environment and can survive in FW (Griffith 1974). Additionally, killifish utilize shallow, warm, low-salinity water for breeding in early spring (Marshall et al. 2003; Marshall et al. 2016). Therefore, our second objective was to investigate whether thermal tolerance increases in response to warm temperature when killifish are in FW. We hypothesized that the physiological adjustments required for acclimation to warm temperature may conflict with maintaining ionic/osmotic homeostasis in FW. For example, acclimation to warm temperature reduces interlamellar cell mass coverage and increases total lamellar surface area (McBryan et al. 2016; Ridgway and Scott 2023). Reflecting the 'osmorespiratory compromise', these morphological adjustments are likely to increase the passive loss of ions, and the gain of water, in FW-acclimated fish, presenting a challenge for ionic/osmotic homeostasis. Therefore, we predicted that thermal tolerance plasticity in response to warm temperature would be either constrained in FW or require compensatory ion/osmoregulatory adjustments. Our results clearly demonstrate that thermal tolerance plasticity in response to warm temperature is not constrained in FW, as fish in both the constant and fluctuating temperature groups exhibited substantial gains in CTmax relative to controls (Fig. 3B).

When looking across studies, our data also indicate that CTmax values of killifish acclimated to FW are generally lower than those of SW-acclimated fish. This result supports the growing evidence that thermal tolerance limits are lower in brackish and marine organisms when they are acclimated to low salinity (Farias et al. 2024). There are several possible explanations for this phenomenon. FW-acclimated killifish are known to adopt gill morphologies that reduce surface area and increase diffusion distance, thereby limiting the passive flux of ions and water (McBryan et al. 2016). If thermal tolerance is influenced by oxygen uptake and utilization, as suggested by the hypothesis of oxygen and capacity limitation of thermal tolerance (OCLTT) in some fishes (Pörtner 2010), then this 'closed' gill morphology may constrain oxygen uptake under highly demanding metabolic conditions, offering a mechanistic basis for the lower CTmax values of FW-acclimated killifish. Alternatively, killifish prefer higher salinities (Fritz and Garside 1974; Bucking et al. 2012; Marshall et al. 2016), and osmoregulation is deemed energetically demanding (Kidder et al. 2006a, b). Thus, more energy may be allocated toward osmoregulation in FW, reducing the energy available for thermal adjustments. Finally, in other fishes, acclimation to high salinity is associated with the elevated expression of heat shock proteins (Deane and Woo 2011). Therefore, improved thermal performance at high salinity may arise from an upregulation of cellular protective mechanisms within tissues. Future studies are clearly warranted to uncover the mechanistic bases for the salinity-dependent nature of thermal tolerance. Our results support the conclusion that, despite salinity-specific differences in upper thermal limits, killifish exhibit a similar capacity for thermal tolerance plasticity in response to warm temperature acclimation in both FW and SW environments.

Warm acclimation impairs osmoregulation upon acute transfer to freshwater

A third objective of our study was to examine the osmoregulatory effects of warm temperature on killifish in FW and SW. When killifish were acclimated to constant and fluctuating warm temperature in SW, we observed a significant treatment*salinity interaction on plasma osmolality. Specifically, acclimation to both constant and fluctuating warm temperature led to reduced plasma osmolality upon SW→FW transfer compared to controls (Fig. 4A), indicating a compromised capacity to maintain ionic/osmotic homeostasis following a hyposmotic challenge. Killifish express Ncc2 in the apical membrane of 'FW-type' ionocytes to facilitate the branchial absorption of Na^+ and Cl^- (Breves et al. 2020). Therefore, impaired hyper-osmoregulatory performance associated with warm temperature acclimation may arise from the differential expression of *ncc2* depending on thermal conditions. However, our data do not support this hypothesis, as we did not observe significant treatment

or interaction effects on *ncc2* expression when killifish were acclimated to warm temperature in SW and then acutely transferred to FW (Fig. 5A). Coping strategies employed by killifish when exposed to FW also involve reducing passive ion diffusion (Marshall et al. 2003). Since warm acclimation is known to reduce interlamellar cell masses and increase lamellar surface area in killifish (McBryan et al. 2016), acclimation to constant and fluctuating warm temperature may have exacerbated diffusive ion loss and water gain, leading to reduced plasma osmolality upon transfer to FW. Furthermore, since the temperature treatments employed in our study fall well within the physiological tolerance limits of killifish (Fangue et al. 2009), the changes in plasma osmolality observed here may be insufficient to initiate the *de novo* synthesis of ion transporters by ionocytes. Taken together, our data suggest that acclimation to warm temperature reduces the hyper-osmoregulatory performance of killifish acutely exposed to FW; however, at least within the experimental conditions studied here, killifish do not respond with compensatory shifts in the activity of branchial ionocytes.

Thermal tolerance plasticity in freshwater is accompanied by shifts in ionocyte gene expression

A key finding of our study is that acclimation to warm temperature in FW is accompanied by changes in branchial *ncc2* gene expression. While our study indicates that temperature has only a modest effect on ionocytes compared to salinity, we observed a significant treatment*salinity interaction on *ncc2* expression when killifish were acclimated to warm temperature in FW (Fig. 6A). This interaction appears to be driven by the modulation of *ncc2* expression in the constant and fluctuating groups relative to controls (Fig. 6A). Since Ncc2 mediates branchial ion absorption in FW (Breves et al. 2020), this pattern is consistent with the notion that killifish need to upregulate ion-absorptive pathways to maintain ionic/osmotic homeostasis in warm FW. Following the logic of the 'osmorespiratory compromise', we predicted that the conflicting demands of osmoregulation and respiration at the gill would either constrain thermal tolerance plasticity in FW or necessitate compensatory shifts in ionic/osmotic regulatory mechanisms. Our data support the conclusion that maintaining thermal tolerance plasticity in FW requires 'FW-type' ionocytes to enhance their ion-absorptive capacities. We also found that acclimation to warm temperature in FW is not associated with significant alterations in *nkcc1* or *cftr1* gene expression (Fig. 6B, C). Killifish maintain the expression of *nkcc1* and *cftr1* in the branchial epithelium even when acclimated to FW, suggesting that 'SW-type' ionocytes remain 'poised' for an abrupt increase in environmental salinity (Karnaky 1986; Breves et al. 2020). In the current study, acclimation to warm temperature did not influence the gene expression of ion transporters expressed by 'SW-type' ionocytes, consistent with the observation that warm temperature did not impact plasma osmolality in fish acutely transferred from FW→SW (Fig. 4B). However, an acute transfer from FW→SW was associated with reductions in hematocrit in warm-acclimated fish (Fig. 4D), which could indicate red blood cell shrinkage in response to hyperosmotic conditions. Given that killifish are renowned for their remarkable salinity tolerance, experimental paradigms employing higher salinities (>35 ppt) may be necessary to observe effects of warm temperature on 'SW-type' ionocytes and acute hypo-osmoregulatory performance.

Conclusions

Our study further resolved the remarkable thermal tolerance plasticity of *Fundulus heteroclitus* by leveraging an experimental approach that incorporated fluctuating temperature conditions and FW acclimation. The ability of killifish to maintain elevated thermal tolerance under fluctuating temperature conditions will likely support their performance when confronted with the increased temperature variability predicted for coastal environments due to climate change. However, our study also suggests that maintaining physiological homeostasis in response to increased temperature may be more challenging for killifish inhabiting FW. This conclusion is supported by our findings, which include: (1) upper thermal tolerance is lower in FW-acclimated fish, (2) acclimation to warm temperature leads to reduced hyper-osmoregulatory capacity, and (3) the plasticity of thermal tolerance in FW, but not in SW, is associated with shifts in ion transporter gene expression. Since acclimation to warm temperature in FW likely increases the energetic costs of respiration and ion/osmoregulation, future studies should explore whether rising environmental temperatures are altering how killifish use dilute microhabitats.

Acknowledgements We are grateful to Dr. Steve McCormick and Amy Regish at the Conte Anadromous Fish Research Center (Turners Falls, MA) for plasma osmolality analysis. Tiffany Yang and Vicky Grechukhina provided valuable laboratory assistance. Artistic image of *Fundulus heteroclitus* was produced by Jack Tom and Cole Strang (Honors Biological Illustration, Western Connecticut State University, Danbury, CT).

Author contributions M.Y.M. conceived and designed research; S.P. performed experiments; M.Y.M., S.P., J.P.B. analyzed data; M.Y.M. and J.P.B. interpreted results of experiments; M.Y.M. prepared figures; M.Y.M. drafted manuscript; M.Y.M. and J.P.B. edited and revised manuscript; M.Y.M., S.P., and J.P.B. approved final version of manuscript.

Funding Funding for this study was provided by the Biology Depart-

ment at Western Connecticut State University and a CSU-AAUP Faculty Research Grant to M.Y.M. and a Skidmore Faculty Development Grant to J.P.B.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All applicable institutional guidelines for the care and use of animals were followed.

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