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Salinity modifies upper thermal tolerance and heat shock protein transcriptional responses in Fundulus heteroclitus

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Abstract

Characterizing the physiological responses of aquatic organisms to increasingly variable and extreme abiotic conditions is important for predicting resilience under future climate scenarios. We examined how acclimation to hypersaline conditions affects the physiological responses of mummichogs (*Fundulus heteroclitus*) to acute heat stress. After acclimating mummichogs to low-salinity (3 ppt) and hypersaline (60 ppt) conditions for ≥ 3 weeks, we assessed upper thermal tolerance using critical thermal maximum (CTmax). We then examined how salinity affected whole-animal physiological responses and heat shock protein (HSP) gene expression in mummichogs subjected to acute heat shock. CTmax was 1.3°C higher in fish acclimated to hypersaline conditions as compared to low-salinity fish. For whole-animal physiological responses, hypersalinity-acclimated fish exhibited perturbation in plasma osmolality during acute heat shock, whereas heat shock elicited an increase in hematocrit in low-salinity fish. Salinity also modified the transcriptional responses of inducible (*hsp70-1* and *hsp70-2*) and constitutive (*hsc70*) HSP genes in an organ-specific manner. In the gill and heart, hypersalinity-acclimated fish had higher baseline *hsc70* expression and, during acute heat shock, blunted induction of inducible HSPs compared with low-salinity fish. In contrast, salinity had little effect on baseline and heat-induced HSP gene expression in skeletal muscle. These patterns indicate that acclimation to hypersaline conditions induces physiological adjustments in the gill and heart, including increased *hsc70* expression, that reduce the need for robust HSP induction during acute heat shock. Together, these findings suggest that salinity-dependent whole-animal and HSP transcriptional responses to acute heat stress are associated with variations in upper thermal limits.

INTRODUCTION

Aquatic organisms inhabiting coastal marine environments have evolved physiological mechanisms to cope with dynamic abiotic conditions, including fluctuations in temperature and salinity (Schulte 2007). However, global climate change is altering the magnitude and timing of these fluctuations, potentially pushing organisms to the limits of their phenotypic plasticity and resilience (Stillman 2019). Estuaries and salt marshes are experiencing rising mean temperatures and greater thermal variability associated with frequent and prolonged marine heat waves (Harley et al. 2006; Hoegh-Guldberg et al. 2010; Doney et al. 2012; Frölicher et al. 2018; Oliver et al. 2018; Venegas et al. 2023). More extreme salinity fluctuations are also occurring, driven by changes in precipitation and freshwater (FW) runoff, rising sea levels, and greater coastal seawater (SW) intrusion (Khojasteh et al. 2021; Costa et al. 2023; Röthig et al. 2023; Feist et al. 2025). Moreover, during periods of high temperatures and reduced FW input, which are expected to become more frequent with climate change, the isolation of tide pools from regular tidal exchange can expose intertidal species to hypersaline conditions (Tweedley et al. 2019). Therefore, the physiological responses of aquatic organisms must be better understood to predict tolerance limits under increasingly variable and extreme abiotic conditions.

Euryhaline teleost fishes maintain ionic and osmotic homeostasis across a wide range of environmental salinities by coordinating branchial, gastrointestinal, and renal functions. In SW, fish are hyposmotic to

their environment and must counterbalance passive water loss and ion gain. This is accomplished by drinking SW, excreting isosmotic urine, and actively secreting Na^+ and Cl^- via 'SW-type' ionocytes in the branchial epithelium (Takei et al. 2014; Shaughnessy and Breves 2025). In FW, fish are hyperosmotic to their environment and must mitigate passive water gain and ion loss. In turn, FW-acclimated fish excrete dilute urine and actively absorb ions through branchial 'FW-type' ionocytes (Hiroi and McCormick 2012; Kovac and Goss 2024). Some euryhaline fishes exhibit exceptional osmoregulatory capacities that enable them to tolerate hypersaline environments, generally defined as salinities ≥ 40 ppt (Gonzalez 2012; Tweedley et al. 2019; Esbaugh 2025). Because such salinities exacerbate threats to hydromineral balance, hypersaline-tolerant species enhance their osmoregulatory capacities by increasing drinking rates, intestinal water absorption, and branchial Na^+ and Cl^- secretion while reducing branchial permeability (Uchida et al. 2000; Gonzalez 2012; Laverty and Skadhauge 2012; Kültz 2015; Ouattara et al. 2024; Tao and Breves 2024; Esbaugh 2025). While the cellular and molecular mechanisms underlying acclimation to FW and SW are well characterized, fewer studies have examined the physiological adjustments that occur when fish are exposed to hypersaline conditions (Gonzalez 2012; Esbaugh 2025). Moreover, how fish respond to other environmental stressors while coping with hypersaline conditions remains unresolved.

While salinity and temperature independently affect fishes (Hochachka and Somero 2002; Hwang et al. 2011), their combined effects on physiological performance remain less understood. Factors such as timing, intensity, and the sequence of exposure to each variable (salinity and temperature) will underlie performance impacts. For example, in some fishes, acute heat shock improves survival during a subsequent osmoregulatory challenge, indicating that transient warm temperatures modify the scope for salinity tolerance (DuBeau et al. 1998; Todgham et al. 2005; Niu et al. 2008). A growing body of evidence indicates that the converse is also true; long-term salinity acclimation modifies the thermal tolerances of a wide range of aquatic species (reviewed by Farias et al. 2024). In several euryhaline fishes, upper thermal tolerance, as measured by critical thermal maximum (CTmax), is higher when fish are acclimated to SW versus FW (Sardella et al. 2008a; Sardella et al. 2008b; Metzger et al. 2016; King and Sardella 2017; Monette et al. 2025; Barany et al. 2026). The effects of salinity on thermal tolerance appears to vary across species and experimental conditions given that other studies report either modest or no effects on CTmax (Haney and Walsh 2003; Hines et al. 2019). Regardless, little is known about how hypersalinity impacts upper thermal tolerance in euryhaline fishes.

Heat shock proteins (HSPs) constitute a highly conserved family of cellular proteins present in a wide variety of organisms. Within this family, HSP70 is a molecular chaperone that facilitates the repair and degradation of damaged or denatured proteins (reviewed by Lindquist 1986; Hightower 1991; Feder and Hofmann 1999). In fishes, HSPs are expressed across multiple organs and play important roles in the cellular stress response to environmental stressors, including temperature and salinity (Iwama et al. 1999; Basu et al. 2002). Salinity changes alter HSP expression in fish (reviewed by Deane and Woo 2011), and several studies have linked HSP expression patterns to thermal tolerance (Nakano and Iwama 2002; Healy and Schulte 2012; Metzger et al. 2016). Because HSPs respond to both thermal and

salinity stress, their expression in specific organs may influence how environmental salinity and thermal tolerance interact.

Our study employs the mummichog (*Fundulus heteroclitus*), an important species in ecosystems along the Atlantic coast of North America that exhibits remarkably broad tolerances to temperature and salinity (Griffith 1974; Kneib 1986; Burnett et al. 2007). Recent work shows that salinity modifies the upper thermal tolerance of mummichogs, with SW-acclimated fish reaching higher CTmax temperatures than FW-acclimated fish (Monette et al. 2025; Barany et al. 2026). However, the physiological mechanisms underlying salinity-dependent variation in thermal tolerance remain poorly understood, and it is entirely unknown whether thermal tolerance is retained under hypersaline conditions. Therefore, our objectives were to determine whether salinity is associated with: 1) variation in upper thermal tolerance, 2) differences in organism-level responses to heat shock, and 3) differences in the transcriptional responses of three HSP genes to heat shock. We also assessed whether the responses of HSP genes to heat shock differed among organs with osmoregulatory functions (e.g., gill) and those with non-osmoregulatory functions (e.g., heart and skeletal muscle). To do this, we acclimated mummichogs to either low-salinity (3 ppt) or hypersaline (60 ppt) conditions for ≥ 3 weeks before examining how salinity affected upper thermal tolerance, measured by CTmax, and indices of whole-animal homeostasis (plasma osmolality, hematocrit, plasma glucose). We also investigated how *hsp70-1*, *hsp70-2*, and *hsc70* expression in gill, heart, and skeletal muscle responded to acute heat shock.

MATERIALS AND METHODS

Animals and acclimation conditions

Mummichog (6.1 ± 0.2 g) of mixed sex from the northern subspecies (*Fundulus heteroclitus macrolepidotus*) were used in this study. Fish were field-collected by Aquatic Research Organisms, Inc. (Hampton, NH) at 42° 55′ 20.9″ N, 70° 51′ 08.9″ W and transported to Western Connecticut State University (Danbury, CT). Fish were maintained in replicate 75-L glass aquaria and acclimated for at least 3 weeks to either low-salinity (3 ppt) or hypersalinity (60 ppt) treatments. *Fundulus* are well known for their exceptional salinity tolerance (Griffith 1974); therefore, to maximize potential differences in thermal tolerance, a target salinity of 60 ppt was selected for the hypersalinity treatment. Although this salinity exceeds normal SW (35 ppt) and the preferred salinity for this species (Fritz and Garside 1974; Bucking et al. 2012; Marshall et al. 2016), it is a condition mummichogs are likely to encounter in their natural environment (Able et al. 2012; Gonzalez 2012) and is well below their physiological tolerance of ~ 120 ppt (Griffith 1974). Low-salinity and hypersalinity treatments were 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 20–23°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 (Tetra, Blacksburg, VA). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Western

Connecticut State University. Temperature and salinity values recorded throughout the acclimation period are presented in Fig. 1.

Upper thermal tolerance

We measured upper thermal tolerance 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 equilibrium is lost. CTmax trials were conducted with a subset of fish from each salinity treatment. CTmax trials were conducted 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 at 24°C and the appropriate acclimation salinity. The water bath was heated at 0.3°C min^{-1} using a copper immersion coil connected to an Isotemp 36TD benchtop unit and circulated with submersible pumps. Individual beakers were continuously aerated and monitored with digital thermometers. During each trial, individual fish were continuously observed, and the 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 salinity treatment.

Acute heat shock

We then examined how salinity acclimation affected indices of whole-animal homeostasis and HSP gene expression in response to acute heat shock. To do this, we transferred subsets of fish from the low-salinity and hypersalinity treatments directly from their acclimation tanks to a glass aquarium containing water at the same salinity at 33°C, the onset temperature for inducing branchial HSPs in *F. heteroclitus* (Fangue et al. 2006). Fish from both salinity treatments were sampled at 0, 30, and 120 min of acute heat shock. 30 and 120 min represent the peak induction times for branchial *hsp70-2* and *hsp70-1*, respectively (Healy et al. 2010). No mortality was observed in either salinity treatment during heat shock.

Sampling of fish tissues and blood analysis

All fish were anesthetized with MS-222, length-measured, and weighed. Blood samples were collected from the severed caudal vein using heparinized 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 filaments, heart, and skeletal muscle were excised, blotted, and immersed in RNAlater Stabilization Solution (Invitrogen) for storage 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, all samples 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, Carlsbad, CA). We employed previously described primer sets for *hsp70-1*, *hsp70-2*, and *hsc70* (Fangue et al. 2006), *18s RNA* (Marshall et al. 2018), and *elongation factor 1α* (*ef1α*) (Scott et al. 2005). Non-specific product amplification and primer-dimer formation were assessed by melt curve analysis. 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 sec and 60°C for 1 min. After confirming that levels did not vary across treatments, *18s RNA* levels were used to normalize target genes for the gill, and *ef1α* levels were used to normalize target genes for the heart and skeletal muscle. 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 (3 ppt, 0 min) gill, heart, and muscle cDNA and included on each plate to calculate PCR efficiencies for target- and reference-gene assays. PCR efficiencies ranged from 94% to 101% across all assays. Relative gene expression ratios between groups are reported as a fold-change from the low-salinity treatment at 0 min.

Statistics

A Student's t-test was used to test for a significant difference in mean CTmax between the low-salinity and hypersalinity treatments. For the acute heat shock experiment, two-way ANOVA was used to test for the main effects of salinity (low, hyper) and time (0, 30, 120 min) on indices of whole-animal homeostasis and HSP gene expression. 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 pairwise comparisons. Statistical analyses were performed using Sigma Plot (version 13). The significance level for all tests was set at $p < 0.05$.

RESULTS

Effect of salinity on upper thermal tolerance

To examine the effect of salinity acclimation on upper thermal tolerance, we measured the CTmax of mummichogs acclimated to low-salinity and hypersalinity conditions (Fig. 2). Mean CTmax of low-

salinity and hypersalinity-acclimated fish was 36.9 ± 0.19°C and 38.2 ± 0.22°C, respectively. CTmax was significantly elevated (+ 1.3°C) in hypersalinity-acclimated fish relative to low-salinity fish (Fig. 2; $p < 0.001$).

Effect of salinity on plasma indices during acute heat shock

To examine the effect of salinity on whole-animal homeostasis during heat stress, plasma osmolality, hematocrit, and plasma glucose were measured in fish acclimated to low-salinity and hypersalinity conditions and sampled at 0, 30, and 120 min of heat shock (Fig. 3). We observed significant main effects of salinity ($p < 0.001$) and time ($p = 0.038$) on plasma osmolality (Fig. 3A). Plasma osmolality was higher in hypersalinity-acclimated fish compared with low-salinity fish across all time-points. For hypersalinity-acclimated fish only, plasma osmolality was significantly elevated at 120 min of heat shock compared with 0 min.

We observed a significant main effect of salinity ($p = 0.014$) and a significant salinity*time interaction ($p = 0.026$) on hematocrit (Fig. 3B). Hematocrit was elevated at 30 and 120 min of heat shock compared with 0 min in low-salinity fish; this pattern was not observed in hypersalinity-acclimated fish. Hematocrit was also significantly higher in low-salinity fish versus hypersalinity fish at 120 min of heat shock.

We observed a significant main effect of time ($p < 0.001$) and a significant salinity*time interaction ($p = 0.046$) on plasma glucose (Fig. 3C). For both salinity treatments, plasma glucose was elevated at 30 and 120 min of heat shock compared with 0 min. At 120 min of heat shock, plasma glucose levels were significantly higher in hypersalinity-acclimated fish than in low-salinity fish.

Effect of salinity on hsp70-1, hsp70-2, and hsc70 expression during acute heat shock

To examine the effect of salinity acclimation on HSP regulation, we examined the transcriptional responses of both inducible (*hsp70-1* and *hsp70-2*) and constitutive (*hsc70*) HSP genes to acute heat shock. In the gill, *hsp70-1* expression showed a significant effect of time ($p < 0.001$) and a significant salinity*time interaction ($p < 0.001$; Fig. 4A). Branchial *hsp70-1* increased at 30 and 120 min of heat shock in both salinity treatments but reached significantly higher levels in low-salinity fish at 120 min. Branchial *hsp70-2* showed significant effects of salinity ($p = 0.006$), time ($p < 0.001$), and a significant salinity*time interaction ($p = 0.012$; Fig. 4B). Similar to *hsp70-1*, branchial *hsp70-2* was elevated at 30 and 120 min of heat shock in both salinity treatments but reached significantly higher levels in low-salinity fish at both time points. In contrast, branchial *hsc70* showed only a significant salinity*time interaction ($p = 0.006$) (Fig. 4C). Branchial *hsc70* increased at 120 min of heat shock in low-salinity fish but was not induced by heat shock in hypersalinity-acclimated fish. In addition, fish acclimated to hypersalinity had significantly higher branchial *hsc70* versus low-salinity fish at 0 min, whereas low-salinity fish had higher levels at 120 min of heat shock.

We observed a significant main effect of time ($p < 0.001$) on *hsp70-1* in the heart (Fig. 4D). Cardiac *hsp70-1* was elevated at 30 and 120 min of heat shock compared with 0 min in both salinity treatments. We observed significant main effects of salinity and time ($p < 0.001$), and a significant salinity*time interaction ($p = 0.047$) on cardiac *hsp70-2* (Fig. 4E). Cardiac *hsp70-2* was elevated at 30 and 120 min of heat shock in both salinity treatments but reached significantly higher levels in low-salinity fish at 30 minutes. For *hsc70*, we observed a significant salinity*time interaction ($p = 0.039$; Fig. 4F). Cardiac *hsc70* increased at 120 min of heat shock in low-salinity fish but was not induced by heat shock in hypersalinity-acclimated fish. In addition, fish acclimated to hypersalinity had significantly higher baseline *hsc70* at 0 min relative to low-salinity fish, similar to the pattern observed in the gill (Fig. 4C).

We observed a significant main effect of time ($p < 0.001$) and a significant salinity*time interaction ($p = 0.032$) on *hsp70-1* in skeletal muscle (Fig. 4G). Muscle *hsp70-1* was elevated at 30 and 120 min of heat shock compared with 0 min in both salinity treatments. For *hsp70-2*, we observed a significant main effect of time ($p < 0.001$; Fig. 4H). Muscle *hsp70-2* exhibited an induction pattern similar to *hsp70-1*, with significant induction at both heat-shock time points, but no significant effect of salinity acclimation. For *hsc70*, we observed a significant main effect of time ($p < 0.001$; Fig. 4I). Muscle *hsc70* was elevated in fish from both salinity treatments at 120 min of heat shock.

DISCUSSION

Thermal tolerance is maintained in hypersaline conditions

For euryhaline fishes, a growing body of evidence indicates that environmental salinity can modify upper thermal limits (Sardella et al. 2008a; Sardella et al. 2008b; Metzger et al. 2016; King and Sardella 2017; Monette et al. 2025; Barany et al. 2026). Specifically, in *F. heteroclitus*, recent work shows that fish acclimated to SW exhibit CTmax values up to 2.7°C higher than those of FW-acclimated fish (Monette et al. 2025; Barany et al. 2026). One potential explanation for these salinity-specific differences in thermal tolerance is that maintaining ion balance in low salinity imposes a substantial energetic cost, thereby constraining thermal limits (Barany et al. 2026). This notion is supported by the decline in CTmax observed when fish are transferred from SW to FW (Barany et al. 2026), as they activate ion-uptake mechanisms to restore homeostasis (Scott et al. 2005; Breves et al. 2020). Conversely, following transfer from FW to SW, CTmax progressively increases and becomes less strongly correlated with indices of ion regulation, such as plasma chloride and branchial Na^+/K^+-ATPase activity (Barany et al. 2026). Based on these patterns, Barany et al. (2026) suggested that SW-acclimated fish maintain greater ionic stability and metabolic flexibility, which may facilitate recovery of thermal tolerance. However, little is known about whether the increased osmoregulatory demands associated with hypersaline conditions constrain upper thermal limits. We found that the mean CTmax of fish acclimated to hypersalinity was 1.3°C higher than fish acclimated to low salinity (Fig. 2). Therefore, despite the osmoregulatory demands imposed by hypersaline conditions, thermal tolerance was maintained under the conditions tested in our study. Our experimental design did not include a salinity intermediate to the low-salinity and hypersaline conditions tested here. Therefore, we cannot directly assess, for instance, the CTmax of hypersaline (60 ppt)- versus

SW (35 ppt)-acclimated fish. Nonetheless, our previous finding that fish acclimated to 35 ppt exhibit a CTmax of ~ 40°C suggests that, under the hypersaline conditions tested here, the CTmax of ~ 38°C is slightly lower than that in normal SW (Monette et al. 2025).

The capacity of mummichogs to maintain thermal tolerance under hypersaline conditions aligns with studies examining whole-organism metabolic responses to environmental salinity. In many euryhaline fishes, metabolic rate remains stable across a wide range of salinities up to 40 ppt (Morgan and Iwama 1999; Kidder et al. 2006; Ern and Esbaugh 2018). However, when exposed to salinities exceeding 40 ppt, several species exhibit reduced metabolic rates (Nordlie et al. 1991; Haney and Nordlie 1997; Swanson 1998; Sardella and Brauner 2008). Together, these findings suggest that the increased osmotic load under hypersaline conditions does not necessarily increase whole-animal metabolic demand and may instead promote greater metabolic flexibility or, in some species, metabolic suppression (Esbaugh 2025). If thermal limits are constrained by the cardiorespiratory system's ability to meet the demand for oxygen uptake and tissue delivery, as proposed by the oxygen- and capacity-limited thermal tolerance (OCLTT) framework (Pörtner 2010), then metabolic suppression could reduce oxygen demands and contribute to maintaining higher CTmax under hypersaline conditions. Studies reporting an inverse relationship between metabolic rate and upper thermal tolerance in mummichogs support this hypothesis. For example, when comparing two locally adapted subpopulations, the southern subspecies (*Fundulus heteroclitus heteroclitus*) exhibits higher CTmax temperatures, but lower routine metabolic rates than the northern subspecies (*Fundulus heteroclitus macrolepidotus*) (Healy and Schulte 2012; McBryan et al. 2016). Due to higher resting metabolic rates, mummichogs from the northern subspecies have a reduced aerobic scope, providing a mechanistic basis for their lower thermal limits (Healy and Schulte 2012). Additional studies show that acclimation to warm temperatures increases CTmax and reduces resting metabolic rate in mummichogs (Fangue et al. 2006; Ridgway and Scott 2023), further supporting a link between thermal tolerance and metabolism in this species.

Morphological features of the branchial epithelium may also underlie salinity-dependent patterns of thermal tolerance. In FW, mummichogs adopt a gill morphology characterized by reduced lamellar surface area, thickened epithelia, and increased interlamellar cell mass (Giacomin et al. 2019). Together, these tissue-level morphological adjustments limit passive ion loss by reducing respiratory surface area and increasing the blood-diffusion distance (Gilmour and Turko 2024). However, this morphology likely impairs oxygen uptake, as described by the 'osmorespiratory compromise' (Wood and Eom 2021). Therefore, impaired branchial oxygen uptake or delivery may contribute to the reduced thermal tolerance of FW-acclimated mummichogs. To limit passive water loss, hypersaline-tolerant species also initiate morphological changes that reduce functional gill surface area (Gonzalez 2012; Laverty and Skadhauge 2012; Kültz 2015; Ouattara et al. 2024; Esbaugh 2025). If mummichogs adopt a similar gill morphology in response to hypersaline conditions, reduced oxygen uptake could markedly impact thermal tolerance. However, our results do not support this hypothesis, as fish acclimated to 60 ppt maintained higher CTmax temperatures than fish acclimated to 3 ppt. Future studies examining the impacts of hypersalinity on metabolic adjustments and gill morphology in *F. heteroclitus* may further elucidate the mechanistic basis for salinity-dependent patterns of thermal tolerance.

Salinity modifies whole-animal responses to heat shock

The second objective of our study was to determine whether salinity acclimation is associated with differences in organism-level responses to acute heat shock. To address this, we exposed mummichogs to a heat shock of 33°C and sampled fish at 0, 30, and 120 minutes. Using this paradigm, we directly compared the physiological responses of fish from each salinity treatment to an equivalent thermal stressor, thereby avoiding confounding effects of the CTmax test itself, in which fish with higher thermal tolerances experience elevated temperatures for longer before reaching CTmax. At 60 ppt, the osmotic gradient across the gill is double that in normal SW (35 ppt), increasing the osmotic load (Gonzalez 2012). Indeed, we found that at baseline (0 min), mummichogs acclimated to 60 ppt had elevated plasma osmolality (413 ± 9.4 mmol/kg) relative to those at 3 ppt (358 ± 8.7 mmol/kg) (Fig. 3A), consistent with previous observations of mummichogs acclimated to hypersalinity (Cozzi et al. 2015). In addition, fish acclimated to hypersalinity experienced significant osmotic perturbation during heat shock, as indicated by increased plasma osmolality, whereas heat shock did not affect plasma osmolality in low-salinity fish. The heat shock-induced rise in plasma osmolality in hypersalinity-acclimated fish was sufficient to induce expression of *cystic fibrosis transmembrane conductance regulator* (*cftr*) (data not shown). Together, these data demonstrate that acclimation to hypersalinity entails a higher osmotic homeostatic set point and a diminished capacity to maintain homeostasis during heat shock. As a result, mummichogs acclimated to hypersaline conditions are simultaneously exposed to both heat stress and osmotic disturbance during heat shock.

While examining salinity-specific effects of heat shock on indicators of whole-animal stress responses, we observed that fish acclimated to 3 ppt exhibited rapid and pronounced increases in hematocrit following heat shock, whereas fish acclimated to 60 ppt showed a trend toward decreased hematocrit (Fig. 3B). Hematocrit increases during a stress response because red blood cells swell and the spleen contracts to increase the number of circulating red blood cells (Wendelaar Bonga 1997). Although red blood cell mobilization may increase the blood's oxygen-carrying capacity to meet metabolic demands, elevated hematocrit also increases blood viscosity (Fletcher and Haedrich 1987), which may increase cardiovascular workload and constrain oxygen delivery. If thermal limits are indeed influenced by tissue oxygen delivery as predicted by the OCLTT hypothesis, elevated hematocrit in response to heat stress may explain, at least in part, the reduced CTmax in low-salinity fish. This hypothesis is supported by findings in brook trout (*Salvelinus fontinalis*) exposed to an osmotic stressor (transfer from FW to SW), which showed that hematocrit increases in parallel with a decrease in CTmax (Shaughnessy and McCormick 2018). However, other studies report only weak associations between hematocrit and thermal tolerance (Wang et al. 2014; Muñoz et al. 2018). Therefore, factors beyond the blood's oxygen-carrying capacity likely also contribute to variation in thermal limits. Mummichogs in both salinity treatments showed elevated plasma glucose at 30 and 120 min of heat shock, consistent with the well-defined hyperglycemic response of fish to environmental stressors (Wendelaar Bonga 1997). Fish acclimated to hypersalinity had significantly higher plasma glucose levels at 120 min of heat shock. Given the concurrent rise in plasma osmolality, this may reflect an osmotic-disturbance-induced

response rather than merely a more robust thermal response. Taken together, our results show that salinity-specific thermal tolerances are associated with distinct whole-animal physiological responses to acute heat stress.

Salinity modifies HSP transcriptional responses to acute heat shock

To determine whether salinity acclimation affects HSP responses, we measured *hsp70-1*, *hsp70-2*, and *hsc70* expression in the gill, heart, and skeletal muscle during an acute heat shock. Previous work on mummichogs shows that these HSP genes exhibit distinct responses to heat stress; *hsp70-1* and *hsp70-2* are strongly inducible, whereas the constitutive heat shock cognate (*hsc70*) shows only modest inducibility (Fangue et al. 2006; Healy et al. 2010). In the gill and heart, fish acclimated to 3 ppt induced all three HSP genes, indicating the recruitment of multiple molecular chaperones in response to heat shock (Fig. 4A–F). Fish acclimated to 60 ppt also exhibited increases in *hsp70-1* and *hsp70-2* relative to baseline (0 min) (Fig. 4A, B, D, E). However, under the same thermal stress, *hsp70-2* induction in the gill and heart was blunted compared with fish acclimated to low salinity (Fig. 4B, E). Acclimation to hypersaline conditions also had a similar effect on the induction of *hsp70-1* in the gill, but not in the heart (Fig. 4A, D). The attenuated induction of HSPs in the gill and heart of fish acclimated to hypersalinity was accompanied by higher baseline *hsc70* expression (Fig. 4C, F). These results align with patterns observed in other euryhaline fishes, where salinity influences the regulation of inducible and constitutive HSPs (Deane and Woo 2004; Metzger et al. 2016). In threespine stickleback (*Gasterosteus aculeatus*), the inducibility of branchial *hsp70-1* in response to heat shock was blunted in fish acclimated to 20 ppt compared with those acclimated to 2 ppt (Metzger et al. 2016). In seabream (*Sparus sarba*), fish acclimated to 50 ppt exhibited higher branchial *hsc70* than fish acclimated to 6 ppt (Deane and Woo 2004). Thus, our current findings, when considered alongside previous studies, demonstrate that salinity modifies HSP responses. Moreover, our findings show that differential HSP responses are associated with salinity-specific differences in thermal tolerance.

Previous work suggests that the magnitude of the HSP response in mummichogs may be linked to variation in thermal tolerance (Fangue et al. 2006; Healy et al. 2010). For example, the southern subspecies of *F. heteroclitus* has a CTmax that is 1.5°C higher than the northern subspecies across a range of acclimation temperatures (Fangue et al. 2006). For the northern subspecies, this lower thermal limit is associated with greater inducibility of branchial *hsp70-2* in response to heat shock (Fangue et al. 2006; Healy et al. 2010). Because the magnitude of the heat shock response is thought to be proportional to the extent of protein damage (DiDomenico et al. 1982), a more robust *hsp70-2* response in the northern subspecies may reflect greater thermal sensitivity in that group (Fangue et al. 2006). In contrast, the association between thermal tolerance and *hsp70-1* expression is less clear; the magnitude of induction in response to heat shock did not differ between northern and southern populations for branchial *hsp70-1* (Fangue et al. 2006). Furthermore, Healy et al. (2010) reported greater inducibility of branchial *hsp70-1* in the more thermotolerant southern fish. In the present study, both branchial *hsp70-1* and *hsp70-2* are induced to higher levels in fish acclimated to low salinity (Fig. 4A, B). Because CTmax of

fish acclimated to 3 ppt was 1.3°C lower than that of fish acclimated to 60 ppt, our results support the hypothesis that a stronger HSP response reflects greater thermal sensitivity in mummichogs acclimated to low salinity.

One possible explanation for the salinity-dependent HSP responses we observed is that acclimation to hypersaline conditions induces physiological adjustments that confer tissue-level protection against heat stress. For example, acclimation to 60 ppt resulted in higher baseline (0 min) *hsc70* expression in both the gill and heart (Fig. 4C, F). As a constitutive molecular chaperone, HSC70 maintains protein homeostasis under normal physiological conditions (Georgopoulos and Welch 1993; Hartl and Hayer-Hartl 2002). Higher baseline *hsc70* expression would help stabilize the protein pool during heat shock, thereby reducing the need for robust induction of other molecular chaperones. Previous findings link *hsc70* to upper thermal tolerance in mummichogs, as fish from the more thermotolerant southern population express higher levels of branchial *hsc70* than those from the northern population (Fangue et al. 2006). Interestingly, HSC70 may also play a role in ionocyte-based Cl^- secretion by mediating the folding, degradation, and trafficking of CFTR (Rubenstein and Zeitlin 2000). Since branchial *cftr1* is upregulated during SW acclimation in mummichogs (Marshall and Singer 2002; Scott et al. 2008; Breves et al. 2024), branchial *hsc70* expression under hypersaline conditions may support the activation of Cl^- transport (Deane and Woo 2011). Ultimately, because branchial *hsc70* expression underlies a shared response to osmotic and thermal stress, its translated product may confer higher thermal limits to mummichogs under hypersaline versus low-salinity conditions.

Our study demonstrates that salinity acclimation modifies HSP transcriptional responses to heat shock in an organ-specific manner. For example, unlike the gill and heart, salinity acclimation had little effect on the inducibility of *hsp70-1* and *hsp70-2* in skeletal muscle and did not increase baseline *hsc70* levels (Fig. 4G, H, I). Given the distinct physiological roles of these organs and their relative proximity to the external environment, organ-specific responses to environmental salinity are not unexpected (Deane and Woo 2004; Todgham et al. 2005; Wang et al. 2007). However, our finding that hypersalinity similarly affects HSP regulation in the gill and heart is particularly interesting, given that the heart is not directly involved in osmoregulation. In mummichogs, previous work linked the capacity to increase heart rate and maintain cardiac rhythmicity to higher CTmax temperatures (Safi et al. 2019). Therefore, because the gill and heart are the main organs responsible for oxygen uptake and delivery, respectively, enhanced cellular protection in these organs may contribute to higher thermal limits in fish acclimated to hypersalinity. To our knowledge, the effects of salinity acclimation on fish cardiac tissue remain largely unexplored. Thus, investigating the molecular and cellular basis of this shared response represents an intriguing avenue for future research.

Conclusions

Our study contributes to a better understanding of how acclimation to hypersaline environments influences organismal performance under heat stress. Specifically, our findings indicate that salinity-dependent patterns of thermal tolerance are associated with distinct organism-level physiological

responses and organ-specific HSP responses to acute heat shock. Although our study cannot establish a direct causal link between HSP transcriptional responses and variation in thermal tolerance, we suggest that acclimation to hypersalinity induces physiological adjustments in the gill and heart that mitigate heat stress. Accordingly, the greater inducibility of HSP genes in response to heat shock in mummichogs acclimated to low salinity may reflect greater cellular stress or protein damage. As climate change alters temperature and salinity dynamics in coastal marine environments, mummichogs exposed to hypersaline conditions for extended durations may maintain higher thermal limits than fish inhabiting low-salinity environments. However, the potential trade-offs associated with hypersalinity acclimation, including effects on other aspects of whole-animal performance and long-term resilience, remain to be explored.

Declarations

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COMPETING INTERESTS

The authors declare that they have no competing interests.

ETHICAL APPROVAL

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

Author Contribution

M.Y.M. conceived and designed research; M.Y.M. performed experiments; M.Y.M. and 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. and J.P.B. approved final version of manuscript.

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Data Availability

Data will be made available upon reasonable request.

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Figures

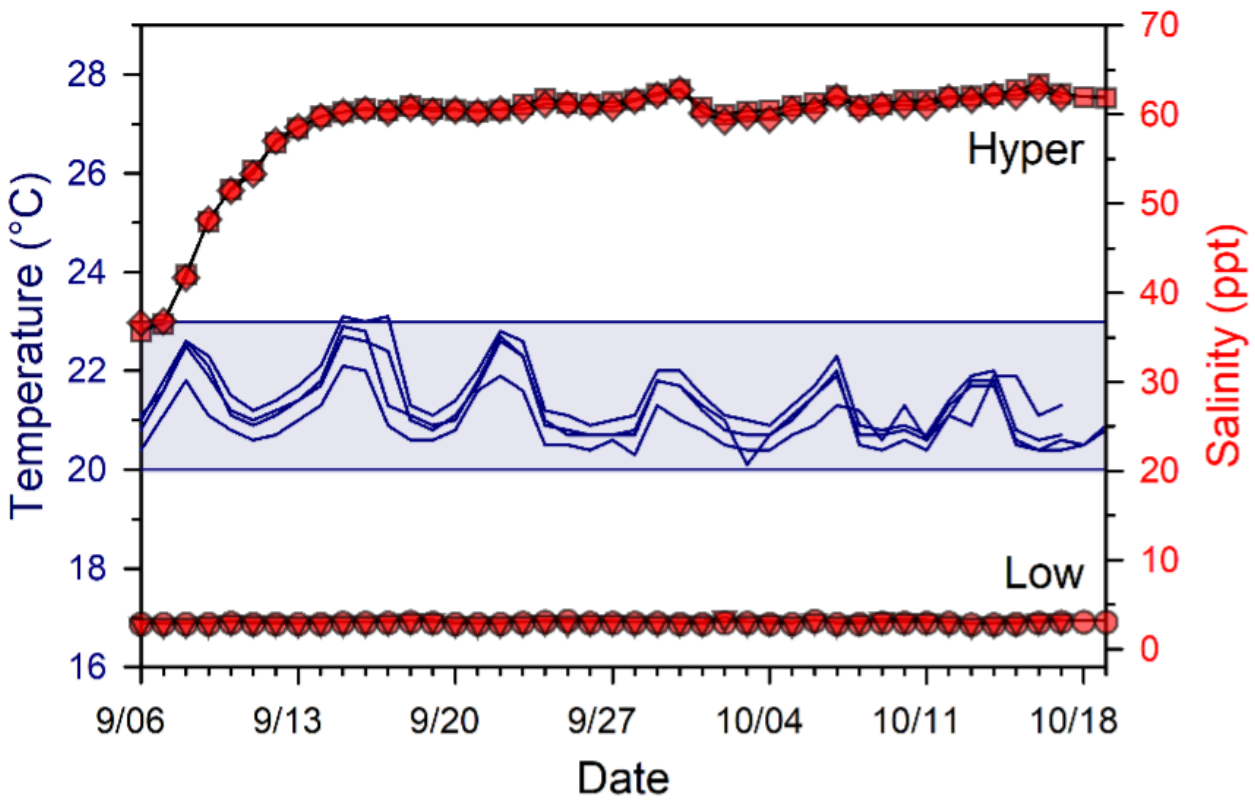


Figure 1

Daily temperature and salinity recordings of low-salinity (3 ppt) and hypersalinity (60 ppt) tanks during the acclimation period. Temperature (°C) is shown on the left y-axis and salinity (ppt) on the right y-axis. Blue lines show temperature measurements from the four acclimation tanks, and the shaded region shows the target temperature range. Red circles and triangles show salinity measurements from the two low-salinity replicate tanks. Red diamonds and squares show salinity measurements from the two hypersalinity replicate tanks. At the start of the study, the hypersalinity tanks were gradually increased from 35 to 60 ppt over an 8-day period.

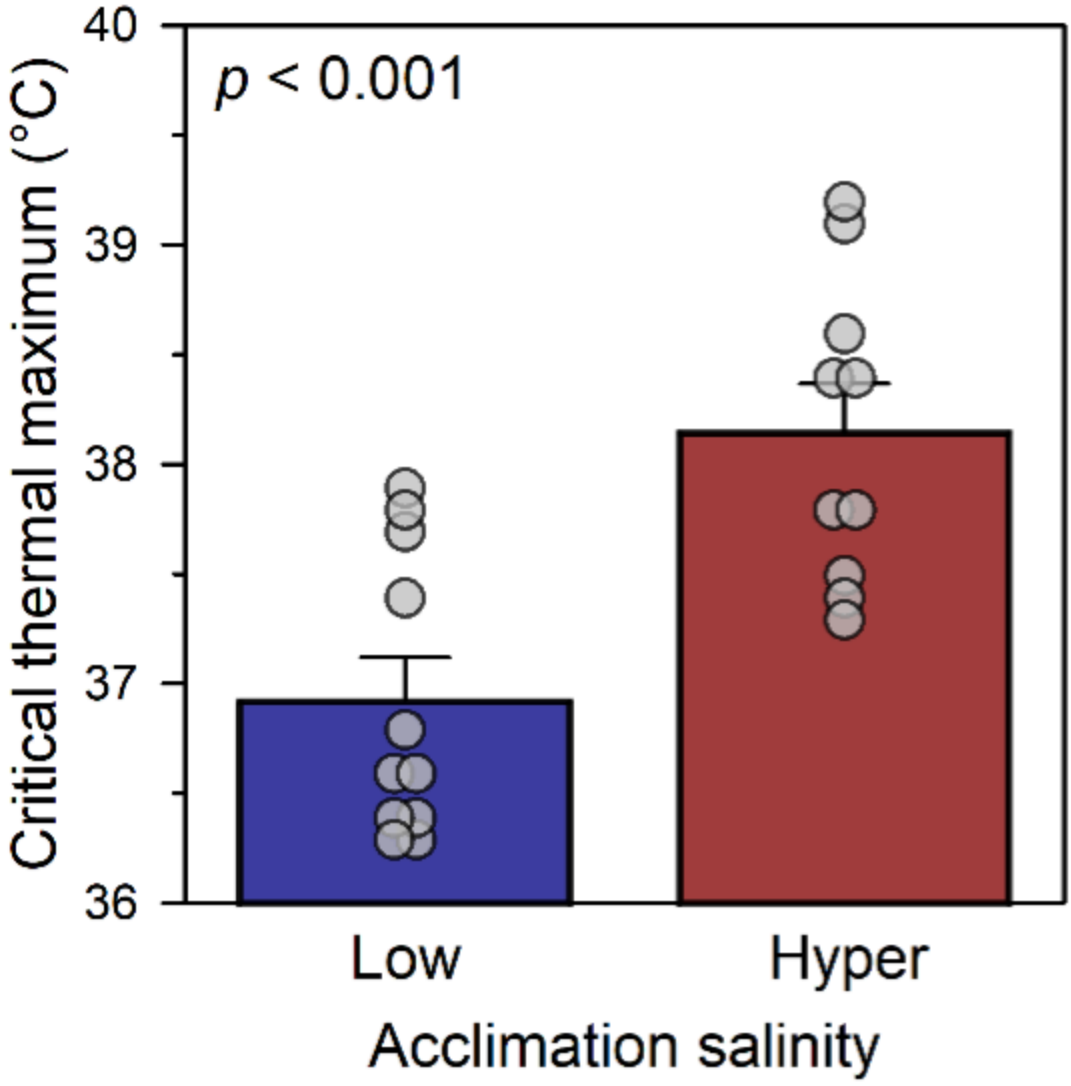


Figure 2

Critical thermal maximum (CTmax) of mummichogs acclimated to low-salinity (3 ppt) or hypersalinity (60 ppt) treatments for ≥3 weeks. Bars represent mean ± s.e.m. (n = 10-11) and circles represent individual values. There was a significant difference (p < 0.001) in mean CTmax between the two salinity treatments (Student's t-test).

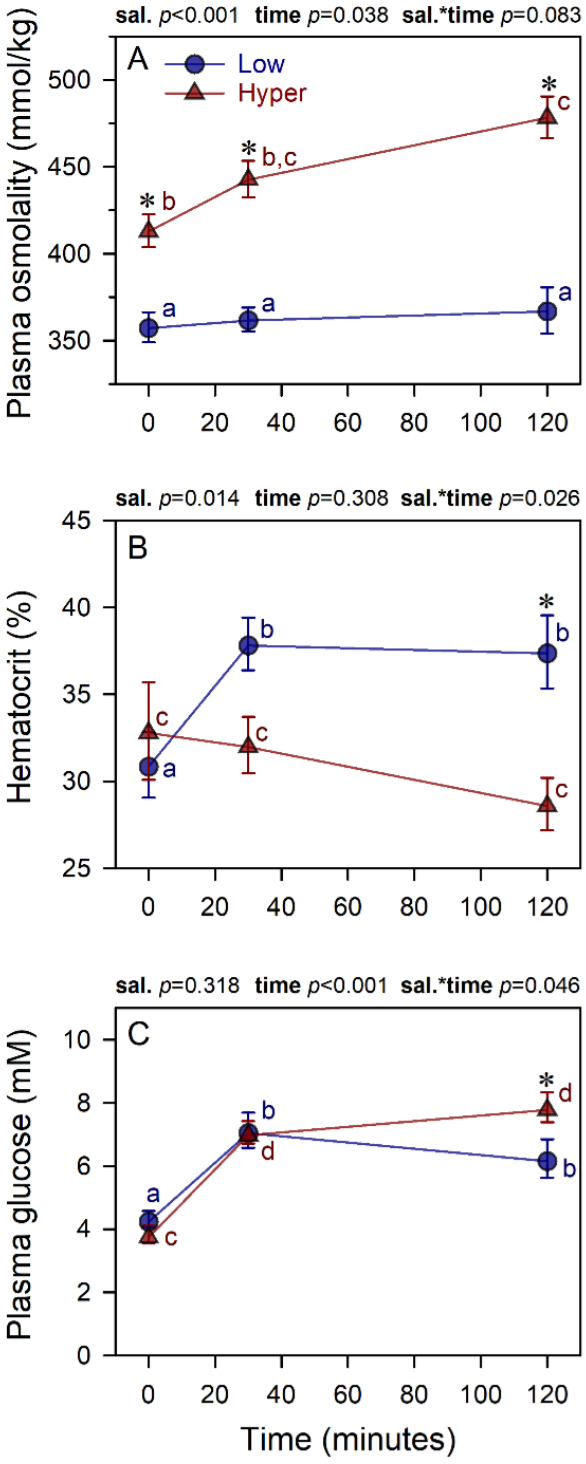


Figure 3

Plasma osmolality (A), hematocrit (B), and plasma glucose (C) of mummichogs acclimated to low-salinity (3 ppt) or hypersalinity (60 ppt) treatments and sampled at 0, 30, and 120 min of acute heat shock (22°C to 33°C). Bars represent mean ± s.e.m. (n = 8-19). Dissimilar letters indicate a significant difference in pairwise comparisons between time points. An * indicates a significant difference ($p < 0.05$) between the low-salinity and hypersalinity treatments within a time point.

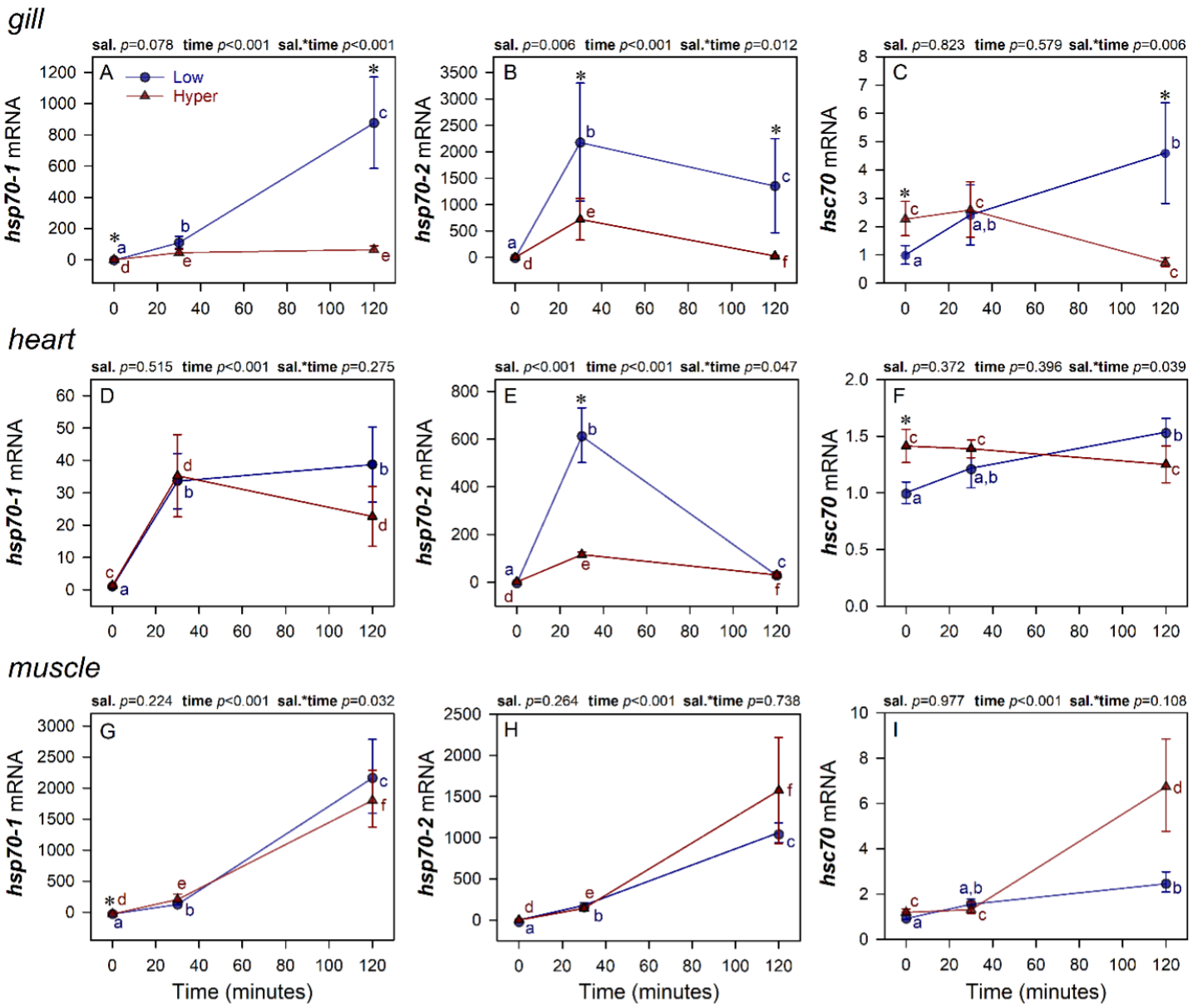


Figure 4

Gene expression of *hsp70-1*, *hsp70-2*, and *hsc70* in gill (A-C), heart (D-F), and skeletal muscle (G-I) of mummichogs acclimated to low-salinity (3 ppt) or hypersalinity (60 ppt) treatments and sampled at 0, 30, and 120 min of acute heat shock (22°C to 33°C). mRNA levels are presented as a fold-change from the low-salinity treatment (0 min). Bars represent mean ± s.e.m. (n = 9-12). Dissimilar letters indicate a significant difference in pairwise comparisons between time points. An * indicates a significant difference (p< 0.05) between the low-salinity and hypersalinity treatments within a time point.