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Effects of Salinity and pH Change on the Physiology of an Estuarine Fish Species, Fundulus heteroclitus heteroclitus

Shauna M. Tietze

EFFECTS OF SALINITY AND pH CHANGE ON THE PHYSIOLOGY OF AN ESTUARINE FISH SPECIES, *FUNDULUS HETEROCLITUS HETEROCLITUS*

by

SHAUNA TIETZE

(Under the Direction of Johanne M. Lewis)

ABSTRACT

Sapelo Island, Georgia is a pristine environment characterized by many variable salt marsh habitats. Throughout the island, salinity ranges from freshwater to full strength seawater and acidity levels range from low pH (pH < 4.4) to above neutral (pH > 8). *Fundulus heteroclitus* (Mummichog) is a resident organism of the salt marsh environment that has evolved coping strategies to withstand fluctuations in temperature, salinity and pH. Field and laboratory studies have shown *F. heteroclitus* to exhibit broad tolerance of individual environmental stressors; however, there is limited information on their ability to handle multiple stressors simultaneously. The goal of this study was to determine if exposure to low salinity and low pH combined is more stressful to *F. heteroclitus* than the factors individually. In a laboratory based experiment, wild caught *F. heteroclitus* were exposed to one of four treatments: ambient (pH 6.8 and 16 ppt salinity); low salinity (2 ppt), low pH (4.5), and low salinity and low pH combined as a multi-stressor treatment. Six fish were sampled from each treatment group at 1, 5, and 7 days post transfer for the analysis of whole-body cortisol as an indicator of stress or rate of oxygen consumption to estimate metabolic costs. In summary, the results indicate cortisol concentration is unaffected by exposure to low salinity and/or low pH at 1, 5, and 7 days post exposure. However, the resting metabolic rate of *F. heteroclitus* increased due to low pH at 1 and 7 days post exposure in both the single and multi-stressor exposures. Increased oxygen consumption is likely due to the elevated ATP demand to facilitate changes at the cellular level in attempt to maintain acid-base balance. These results suggest that *F. heteroclitus* exposed to low pH in natural environments may exhibit energetic tradeoffs that could affect their overall fitness.

INDEX WORDS: *Metabolism, Respirometry, Cortisol, Killifish, Stress, Sapelo Island*

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by

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EFFECTS OF VARYING pH AND SALINITY CHANGE ON THE PHYSIOLOGY OF AN ESTUARINE FISH SPECIES, *FUNDULUS HETEROCLITUS HETEROCLITUS*

By

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DEDICATION

This thesis is dedicated to my Dad, Mike Tietze, for instilling in me his passion for nature and curiosity of the universe;

To my Mom, Shelly Tietze, for always being my #1 fan and providing unconditional love and support throughout my educational journey;

And to my Nana, Dianne Tietze, for always believing in me, encouraging me to pursue my dreams, and showing me what it means to be strong.

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INTRODUCTION

Stress and Stress Response

Stress is a condition in which intrinsic or extrinsic factors (stressors) threaten the homeostasis of an organism. Exposure to a stressor typically results in a stress response, which is the recruitment of a suite of behavioral and physiological responses used by the organism to compensate for the change (Wendelaar Bonga, 1997; Schulte 2014). The stress response of an organism can be categorized into primary, secondary, and tertiary events (Figure 1). The primary stress response involves the release of catecholamines and the stimulation of the hypothalamic-pituitary-interrenal (HPI) axis resulting in the production of corticosteroid hormones (Donaldson 1981; Wendelaar Bonga 1997). Catecholamines are released and degraded within minutes of stressor exposure, whereas corticosteroids, such as cortisol, are released more slowly and may remain elevated for extended periods of time (Lataretu et al., 2013). As such, corticosteroids are commonly measured as an indicator of stress in fishes and other vertebrates (Barton 2002; Kijewska et al., 2016; Mommsen et al., 1999). The release of cortisol triggers secondary responses at the cellular level, such as alterations to plasma and tissue ions and the expression of stress proteins, that aid in acclimation to environmental stressors (Mommsen et al., 1999; Pickering 1981; Vijayan et al., 1997). Secondary responses are energetically costly and if the stressor persists the increased energy expenditure may lead to tertiary responses which can have consequences on the fitness and survival of the organisms (Mommsen et al., 1999).

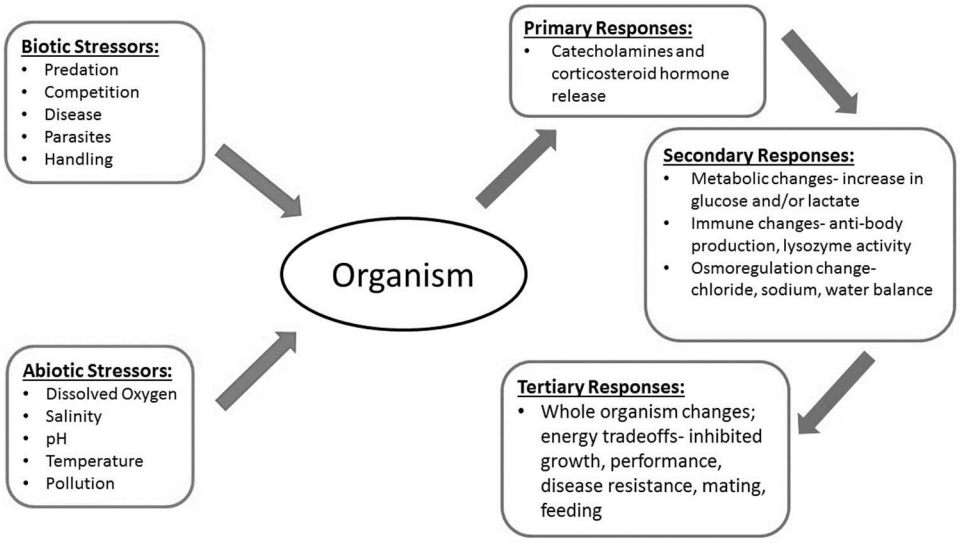

Figure 1. Process of stressor detection leading to the three categories of stress responses. Figure adapted from Barton et al. 2002.

Multi-stressor Environments

Many studies that examine the organismal stress response focus on a single environmental stressor, such as salinity or temperature (Altinok and Grizzle 2003; Ern et al., 2014). However, measuring single stressors may underestimate the level of stress experienced in natural environments where organisms are exposed to fluctuations in multiple environmental factors simultaneously. Changes in a single environmental variable may not be as stressful to an organism when acting alone, but when combined with one or more other variables the interacting effects may create challenges for the organism. The costs of dealing with two or more stressors may be additive, synergistic, or antagonistic (Figure 2). The effect of additive stressors is simply the sum of each individual stressor's effects combined. Synergistic stressors, however, interact to create an effect greater than the sum of the two stressors. Antagonistic stressors are less common; this is when two stressors have a lesser effect when combined. Additive stressors can create minor effects on performance,

whereas synergistic stressors can have substantial effects and may cause unpredictable physiological and behavioral responses (Todgham and Stillman 2013).

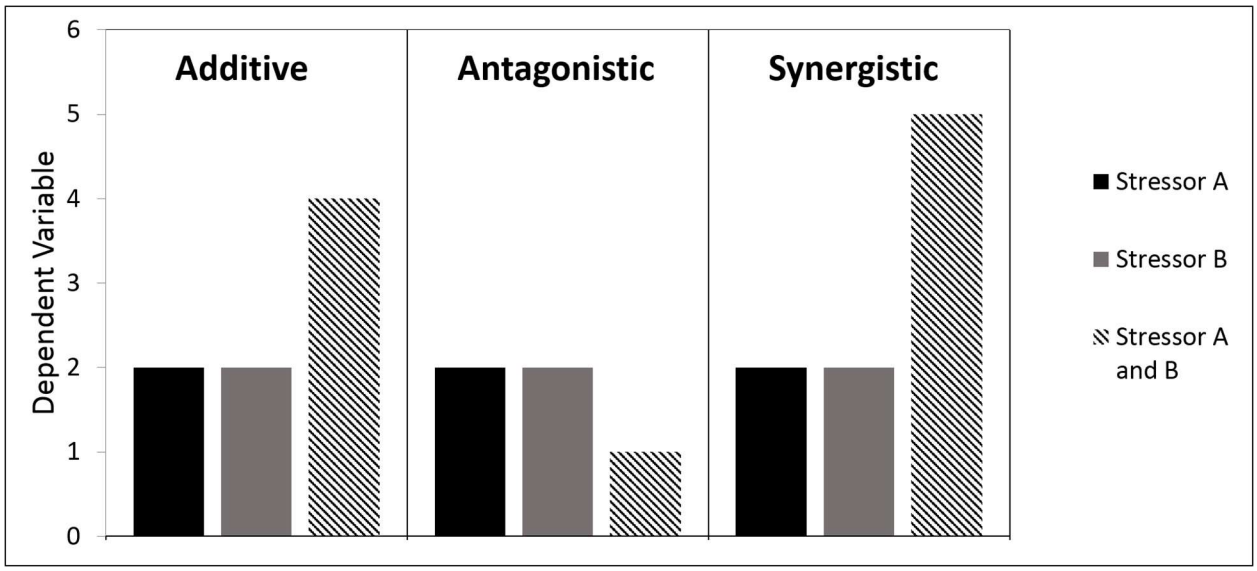

Figure 2. Example of the three possible effects interactive stressors (A and B) may have on the dependent variable indicating performance of the organism. Figure adapted from Todgham and Stillman, 2013.

Multiple stressors can lead to complex interactions, such as the interacting effects of temperature and hypoxia in multiple fish species (Healy et al., 2010; McBryan et al., 2013). McBryan 2012 demonstrated that temperature may not be a substantial stressor independent of hypoxia and vice versa, but when combined the stressors have a synergistic effect on adaptation capability of the fish. Therefore, studies only looking temperature or hypoxia independently may underestimate the magnitude of the stress response elicited by multiple stressor in a natural habitat (Barton 2002; McBryan et al., 2013; Whitehead 2013).

Salt Marshes as Multi-stressor Environments

Salt marshes are the transition zone between land and ocean characterized by grasses and inundation of seawater. They are one of the most complex and productive ecosystems in the world (Weis and Butler 2009). Variations in temperature, dissolved oxygen, salinity, and pH occur naturally due to tidal influence and govern the activity and distribution of organisms living within (Weis and Butler 2009). Within the North American Atlantic Coast salt marshes salinity can range from less than 1 ppt to 35 ppt (seawater) (Weigert 1990). Salinity, pH, and oxygen can vary depending on three main factors: locationally on a gradient depending on the volume of freshwater and saltwater influx, temporally due to tidal fluctuations (high and low tide alternating every six hours, as well as monthly extremes during spring and neap tides), and with unpredictable natural events such as rainfall and drought. In addition to environmental variation, inland and coastal waters in the Southeastern U.S. often have low pH levels due to high organic matter which presents a unique challenge to organisms compared to those living in Northeastern salt marshes (Wiegert et al., 1981). These environmental variables can act as stressors and are a driving force behind selection of behavioral and physiological adaptations seen in resident marsh organisms that allow them to acclimate to such a variable environment (Angilletta and Sears 2011).

Adaptations in Salt Marsh Fishes

Euryhalinity is a rare adaptation exhibited by only 2% of ray finned fishes, most of which live in salt marsh habitats (Schultz and McCormick 2013). This adaptation allows fishes to tolerate wide ranges in salinity (from freshwater to full seawater) to find food, reduce parasite load, and deal with natural salinity changes such as tide and rainfall. Although advantageous, euryhalinity alters the amount of energy a fish is able to expend on feeding, mating, and predator avoidance (Altinok

and Grizzle 2003; Febry and Lutz 1987). To reduce the cost of maintaining homeostasis with respect to ion and water balance, some fishes exhibit behavioral adaptations, such as drinking more water to increase excretion of excess salts in high salinity areas (Evans 1993) or simply avoiding areas of disfavored salinity (Tietze and Gerald 2016). When fishes are faced with a salinity challenge, a primary stress response is activated, such as cortisol release which triggers mechanisms that aid in maintaining homeostasis (Barton and Iwama 1991). Bodily fluids of fishes in freshwater are hyperosmotic to the external environment, therefore they must counteract the gain of water and loss of NaCl by excreting large volumes of dilute urine and activate NaCl uptake within their gills. However, in a saltwater environment the fish's bodily fluids are hyposmotic to the environment and must reduce the loss of water and gain of NaCl by ingesting seawater and excretion small volumes of ion-concentrated urine as well as active secretion of NaCl through the gill epithelium (Evans 2008) (Figure 3).

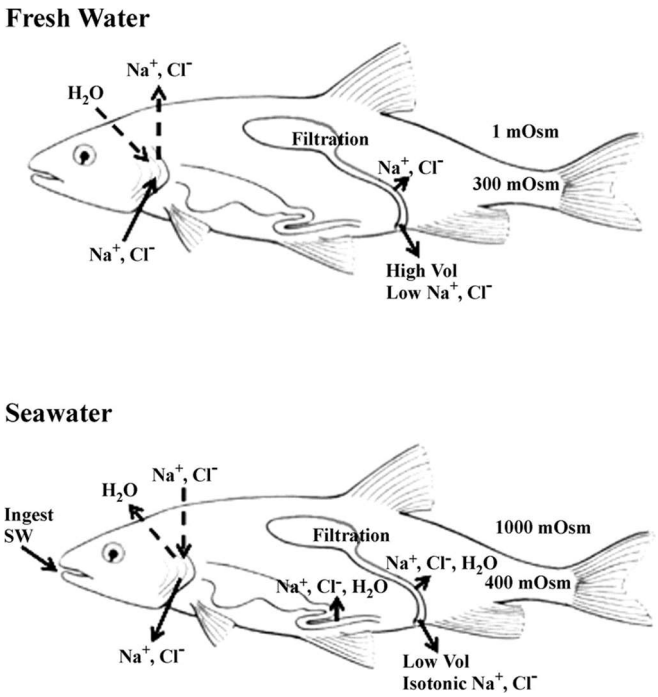

Figure 3. Mechanisms of osmoregulation by teleost fishes in freshwater and seawater environments in freshwater, fishes prevent the net gain of water and loss of salt by excreting large amounts of dilute urine and increasing ion absorption. Whereas is saltwater, fishes prevent the net gain of salt and loss of water by drinking seawater and excreting salt through urine and active processes in the gills. Passive ion movements denoted by dashed arrows; active by solid arrows. Figure used with permission from Evans 2008.

Most salt marsh fishes have evolved secondary stress response mechanisms that facilitate ion balance by altering the concentration of transport proteins to alter ion flux rate (Cerdà and Finn 2010; Evans et al., 2005; Jung et al., 2012; Tipsmark et al., 2002). Ion flux is regulated by the gill epithelium to maintain ion balance in response to environmental conditions and is especially important for fishes living in tidal marshes (Scott et al., 2004a). Secretion of ions through the gills of most euryhaline fishes is driven by the formation of an electrochemical gradient facilitating ion movement through active transport (Scott et al., 2004a). Ion transport is facilitated by mitochondria-rich cells in the epithelium which either absorb or secrete ions depending on the environment (Katoh et al., 2003). Changes in cell volume also help regulate ion transport activity in the gills. Cell shrinkage is linked to an increase in Cl^- secretion occurs when a fish is in high

salinity. A decrease in Cl^- secretion is linked to cell swelling and occurs when a fish is in low salinity (Kültz 2005; Scott et al., 2005).

Non-adapted fishes living in acidic water, normally due to pollution, experience increased number and turnover rate of ionocytes, increased mucus production possibly leading to suffocation, and break down of gill tissue (Laurent and Perry 1991). However, some marsh species have evolved adaptations to live in low pH environments. These fishes regulate body pH by production and excretion of H^+ ions and by manipulating Na^+ and Cl^- influx through Na^+/H^+ and HCO_3^-/Cl^- exchange mechanisms in cell membranes (Wood and Marshall 1994). Some fishes, such as *Fundulus heteroclitus,* have evolved adaptations beyond that of other fishes in order with withstand low pH environments, such as the ability to alter Na^+ and Cl^- efflux rates without altering influx rates to maintain ionic homeostasis.

Many studies have described the coping mechanisms of salt marsh organisms to the individual challenges of salinity or pH. However, these single stressor studies may underestimate the pressures that are placed on the organisms in the natural environment. Research assessing the responses of organisms when exposed to multiple stressors present in salt marshes, such as varying salinity and pH, will provide more realistic view on the interactions between stressors as well as the physiological mechanisms animals utilize in natural environments.

Fundulus heteroclitus

The most common salt marsh fish on the North American Atlantic Coast is *Fundulus heteroclitus* (Lineaus 1766) (Able et al., 2012), a hardy fish that has evolved coping strategies allowing them

to live highly variable environments and making them an ideal organism on which to study multi-stressor effects (Schulte 2014). Geographic differences in environmental stressors has led to two subspecies of *F. heteroclitus* which exhibit variation in morphology, genetics, behavior, and physiology (Picard and Schulte 2004; Scott et al., 2004b; Sloman 2004; Whitehead et al., 2012). The northern subspecies*, F. h. macrolepidotus* (Walbaum, 1792)*,* range from Nova Scotia to New Jersey and have adapted to and prefer low salinity near freshwater (Fangue et al., 2006). However, the southern subspecies, *F. h. heteroclitus,* range from the southern Chesapeake Bay to Florida and prefer brackish to full strength seawater (10 – 35 ppt) (Bucking et al., 2012; Fangue et al., 2006). The two populations overlap in the Chesapeake Bay, however the southern population inhabits the coastal waters while the northern population inhabits freshwater (Schulte 2007). Supporting these geographical observations, the northern population is much more tolerant of freshwater in laboratory experiments as well. When transferred to freshwater, 20% of the southern population experience mortality; while the northern population experiences no significant mortality (Scott et al., 2004b). The mortality rates between populations was associated with inability of the southern fish to activate the Na^+/K^+-ATPase in order to regulate plasma Na^+ and Cl^- levels. The process of salinity acclimation is mediated by primary stress response mechanisms, such as cortisol release (Scott et al., 2005), which differs between the two subspecies and may be the reason that the northern population can colonize freshwater while the southern population cannot (DeKoning et al., 2004). Cortisol concentration, which is an early indicator of stress in fishes (McCormick 2001), is higher in the northern population at rest, but higher in the southern population under stress (DeKoning et al., 2004).

Although the southern subspecies of *F. heteroclitus* experience mortality in pure freshwater laboratory experiments, they exhibit a broad salinity tolerance range and are able to maintain ionic homeostasis from near freshwater (1 ppt) up to four times the strength of seawater (140 ppt). If *F. heteroclitus* stay in low salinity longer than the length of a tidal cycle, they are able to transition to a freshwater gill type (Copeland 1950; Philpott and Copeland 1963; Whitehead et al., 2012). This transition only occurs completely in the northern subspecies and partially in the southern subspecies if there is no option to avoid low salinity (Schulte 2014). The process of changing gill type requires oxygen for cellular respiration to facilitate ATP-ase pumps. The energetic cost of this process can have negative interacting effects on physiology and behavior (Swanson 1998). The incurred cost is often measured by oxygen consumption as an indication of metabolic rate. Twenty-five to fifty percent of a fish's total metabolic energy can be put towards osmoregulation (Iwama et al., 1999).

F. heteroclitus also exhibit a tolerance for pH as low as 3.75 (Gonzalez et al., 1989), which allows them to live in acidic salt marsh habitats in the Southeastern U.S. Atlantic Coast. As mentioned previously, most fishes regulate acid/base concentrations by altering H^+ production and excretion; however, Patrick and Wood (1999) have shown that *F. heteroclitus* also compensate in acidic environments by simultaneously reducing Na^+ efflux and initiating Cl^- efflux without altering either Na^+ or Cl^- influx rates. Na^+ uptake and H^+ efflux in freshwater can be inhibited by low pH which suggests that low salinity and low pH combined may increase stress response on *F. heteroclitus* (Patrick and Wood 1999). This reality of multi-stressor challenges that *F. heteroclitus* face in nature is not yet well studied, especially in southern populations as most studies have been on the northern populations.

Study Objectives

F. heteroclitus that inhabit Southeastern U.S. Atlantic salt marshes are regularly exposed to tidal and weather related salinity fluctuations and also simultaneously experience low pH (<4.4) in some habitats (Chalmers 1997). While effects of pH and salinity on *F. heteroclitus* have been examined individually, interactive effects are less understood. To address this area of interest, I performed a laboratory study to investigate both the individual and combined physiological effects of altered salinity and pH on a brackish water population of *F. heteroclitus* from Sapelo Island, GA. This was accomplished by analyzing markers of stress response and energetic costs associated with acute (1 day) and chronic (5-7 days) exposure to low salinity and/or low pH. Stress response was determined by whole-body cortisol and energetic cost was determined by oxygen consumption as an estimate of resting metabolic rate. I hypothesized that the multi-stressor environment would elicit a stronger stress response and higher energetic cost than the single stressor environments (Figure 4) with the highest levels at acute exposure time and decreasing overtime as the fish acclimated to its environment.

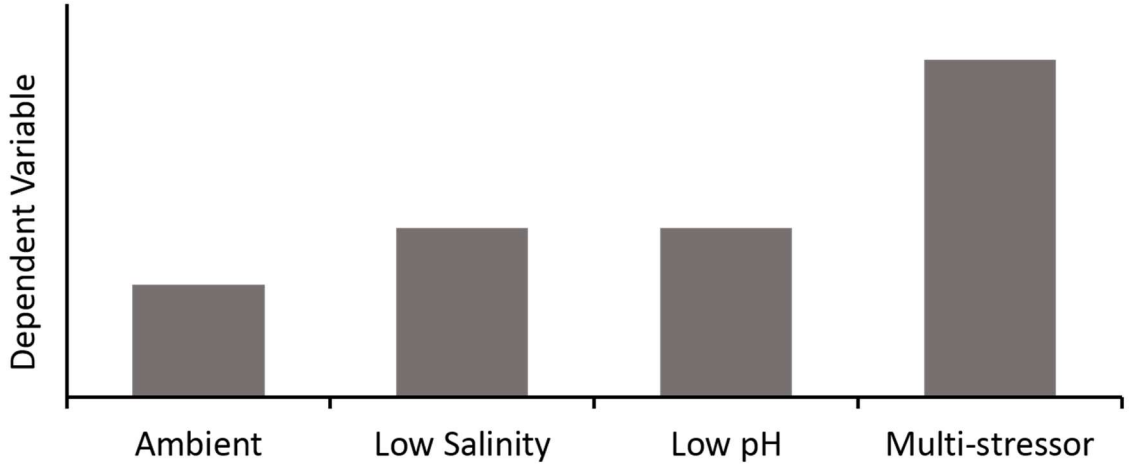

Figure 4. Prediction of treatment effect on dependent variable (cortisol concentration or oxygen consumption rate) respectively.

METHODOLOGY

Study Site: Sapelo Island, GA

Salt marshes on Sapelo Island, GA, U.S.A are an ideal study system to examine the effects of living in a multi-stressor environment on resident organisms. The wetland areas of the island are classified as salt marsh estuary due to of the lack of freshwater input by rivers (Howard and Frey 1985). The only freshwater inputs are rainfall, runoff, and groundwater discharge (Chalmers 1997). Approximately 80% of aquatic habitats on Sapelo Island are intertidal marsh and mud flats, and the remaining is permanently submerged (Chalmers 1997). The island's tidal creeks have a uniquely wide range of pH and salinity. Personal measurements have shown pH levels from 4.4 to 8.6, and salinities from 0 ppt (freshwater) to 35 ppt (seawater) in mudflats, streams, and ponds. These variable conditions require resident organisms to have specific adaptations to maintain homeostasis and also make this environment an ideal model system to access the effects of multiple stressors, specifically pH and salinity, on aquatic organisms.

Fish Collection & Husbandry

All experimental protocols were approved by the Georgia Southern University Animal Care Committee (IACUC #I15013). Fish collection was permitted under the Georgia Department of Natural Resources (permit #8926).

Two hundred *F. heteroclitus* were captured using dog-food baited minnow traps from a tidally influenced culvert stream on Sapelo Island (15-18 ppt salinity; 6.7-6.9 pH). Fish were transported back in aerated coolers to Georgia Southern University and held in a 378 L tank filled with reverse osmosis filtered tap water and a carbon filtration system. Calcium chloride and sodium

bicarbonate were added to all water treatments at a concentration of 100 g L^{-1} to replenish ions removed from the R.O. filtration. The temperature of the tank was approximately 20° C and maintained in a room with a 14:10 light:dark cycle. Salinity ranged from 15-17 ppt during the holding period using Instant Ocean® Aquarium Salt. The pH ranged from 6.7-6.9 using additions of hydrochloric acid (HCl). Salinity and pH were measured daily and adjusted as needed. Water adjustments were conducted in stock water prior to addition to the tank to minimize stress to the fish. Levels of ammonia (< 5 ppm), nitrate (< 40 ppm), and nitrite (< 1.0 ppm) were measured daily using aquarium test kits. If measurements exceeded the aforementioned limits, a 50% water change was performed. Otherwise, 25% of the water was changed every other day. Fish were fed commercial cichlid pellets daily to satiation. All fish were acclimated to capture site (ambient) conditions (16 ppt, 6.8 pH) for at least 14 days before trials began. Average fish weight was 5.14 g ± 0.42 for experiment 1 and 7.6 g ± 0.19 for experiment 2.

Fish and Water Treatments for Experiments 1 & 2

Fish were subjected to four different water treatments for 7 days in order to analyze whole-body cortisol and gene expression in experiment 1 and oxygen consumption in experiment 2 (Table 1.1)

Table 1. Salinity and pH levels of the four treatment groups: ambient (same as capture site; 16 ppt, 6.8 pH), low salinity (2 ppt, 6.8 pH), low pH (16 ppt, 4.5 pH), and multi-stressor (2 ppt, 4.5 pH).

		Salinity	
		Moderate (16 ppt)	Low (2 ppt)
pH	Neutral (6.8)	Ambient	Low Salinity
	Low (4.5)	Low pH	Multi-stressor

Experiment 1: Analysis of Cortisol Response & Gene Expression

Following acclimation, *F. heteroclitus* were haphazardly assigned to one of the four treatments (ambient, low pH, low salinity, or multi-stressor) and then divided evenly between three replicate tanks per treatment (18 fish per treatment, 6 fish per tank). Replicate tanks were used to test for any unknown variability between tanks within a treatment. Salinity and pH were measured twice daily and at every sampling event and adjusted accordingly to maintain salinity within 16 ppt ± 1 and pH of 6.8 ± 0.1.

Two fish per tank (n = 6) were sampled 1, 5, and 7 days post treatment exposure. Sampling events were performed at approximately the same time each day (10 a.m.). Body weight, standard length, and total length were recorded for each sampled fish. Sampled fish were euthanized with a lethal dosage of 150 mg L^{-1} tricaine mesylate (MS-222, buffered 1:2 with $NaHCO_3$) in a process lasting 2-3 minutes. Gills were extracted from the fish immediately following death and flash frozen in liquid nitrogen along with the rest of the body. Samples were then stored at -80°C until analysis.

Cortisol

Whole frozen *F. heteroclitus* were powdered over liquid nitrogen and homogenized on ice in 500 µl of 1X PBS (800mL; 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4; pH 7.4) with an electric homogenizer (PowerGen 125, Fisher Scientific, Waltham, MA, USA). The homogenizer was rinsed with an additional 500 µl of 1X PBS into the tube containing the homogenate. Cortisol was extracted from the homogenate using a protocol adapted from Jeffrey & Gilmour (2016). In summary, 2 mL of ethyl acetate was added to each sample and vortexed for 1 minute then centrifuged at 3,500 RPM at 4°C for 5 minutes. The aqueous phase supernatant was then transferred to a new tube and the extraction with ethyl acetate was repeated another 2 times. Next, 1600 µl of supernatant was transferred to a 10 mL glass tube and evaporated using nitrogen gas. Extracts were dissolved in 1 mL of 1X extraction buffer (Neogen cortisol enzyme-linked immunosorbant assay (ELISA) kit, Lexington, KY, USA) and incubated overnight at 4°C. Cortisol levels were measured in duplicates using an ELISA kit (Neogen, Lexington, KY, USA) and measured on a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 650 nm at 22°C. Concentrations of samples were determined by comparing the binding efficiency to serially diluted cortisol standards. The extraction protocol for whole-body cortisol extraction was validated for the laboratory (Boykin 2016). Efficiency was analyzed by spiking non-experimental homogenates with 2 µl of 10 ng mL^{-1} of cortisol stock solution (Sigma Aldrich). The extraction process was performed identically for spiked samples as for non-spiked samples and the efficiency was determined based on the ability to effectively recover the known amount of cortisol stock solution using the protocol. The average extraction efficiency was 94.3%, which was within the accepted range in previous studies that examined whole-body cortisol levels (Ramsay et al., 2006).

Experiment 2: Analysis of Oxygen Consumption Rate with Intermittent-flow Respirometry

Intermittent-flow respirometry was used to measure the effect of acute (1 day) and chronic (7 days) exposure to the four experimental treatments described previously (ambient, low salinity, low pH, and multi-stressor) on an individual's oxygen consumption rate (MO_2; mg O_2 kg^{-1} h^{-1}). As oxygen consumption rates were obtained from mature fish in a resting state that were 1 day post feeding, the measurement represents the fish's resting metabolic rate (RMR) (Rosewarne et al., 2016). Fish were measured individually in the oxygen chamber and housed as pairs in 75 L tanks treatment tanks when not being measured. All fish were placed in the chamber 12 hours before measurement to acclimate to the chamber and minimize exploratory behavior (Rosewarne et al., 2016; Svendsen et al., 2016). All fish were weighed and measured following each trial allowing fish to be distinguished from one another by standard and total length.

Setup

The intermittent respirometry protocol implemented in this project was modified from Rosewarne et al. (2016) and Svendsen et al. (2016). The respirometry system consisted of a 375 ml chamber that held the fish, a flush pump, a recirculation pump, 85 cm tubing, and a small flow through chamber that housed the galvanic oxygen probe (ADInstruments) (Figure 5). The chamber was submerged in 37 L of water to provide continuous aeration to the system during acclimation under ambient temperature. The oxygen probe was connected to a Powerlab 26T computer (AdInstruments) and the digital output was analyzed using the LabChart 7 software (AdInstruments). Respirometer size (375 ml) was chosen based on the average size of the fish (7.6 g ± .19) and expected oxygen consumption rates (Svendsen et al., 2016).

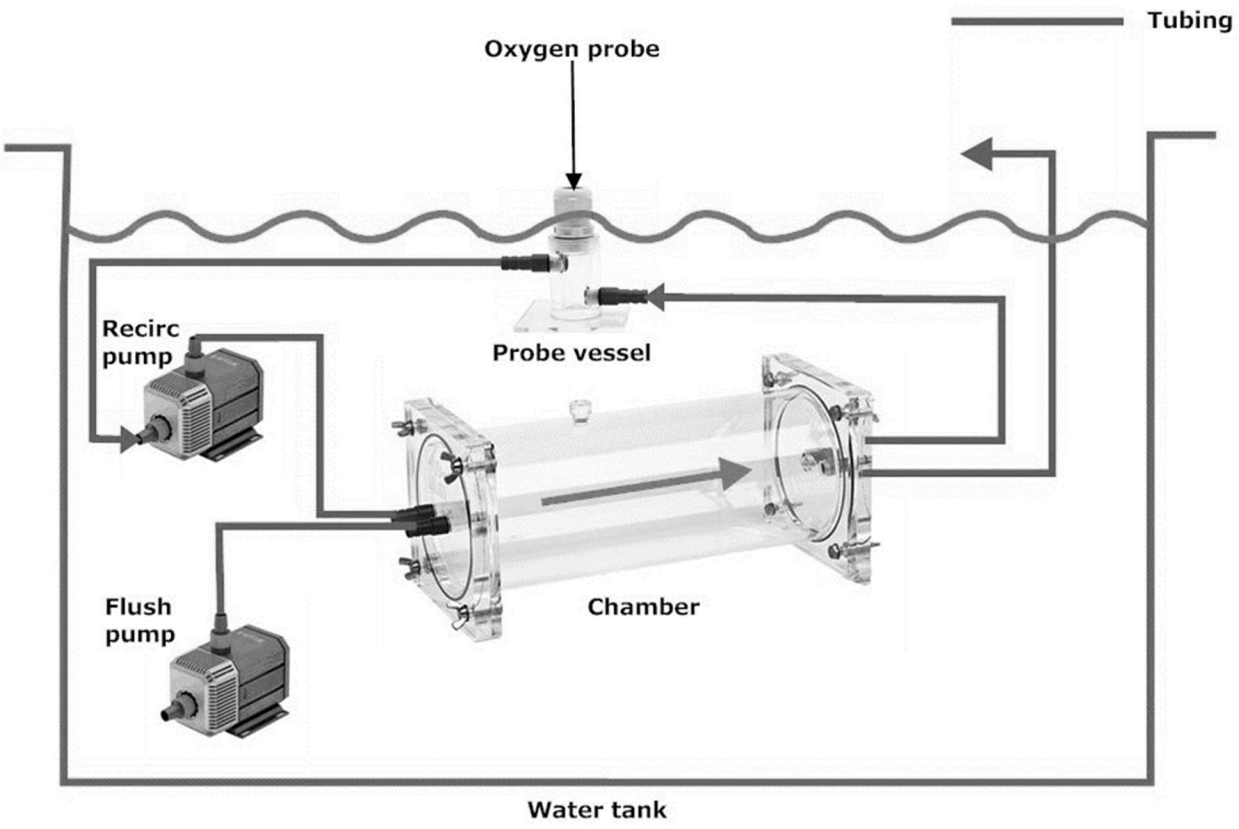

Figure 5. Diagram of the respirometry system submerged in a tank of oxygenated water. Shown is the chamber the fish is held in connected by tubing to a vessel that holds the oxygen probe connected to a computer. Together these items and the recirculation pump make a closed system. The flush pump allows freshly oxygenated water into the system. During the measurement period the system is closed by turning the flush pump off and the recirculation pump on. Only the water within the system is recirculated past the oxygen probe and the decline in oxygen is measured. Figure used with permission from Loligo Systems (2016).

Oxygen Consumption Measurements

Each measurement period consisted of a four-minute flush, a two-minute wait, and a ten-minute measurement (Figure 6). This period was repeated three consecutive times per fish. The flush period allowed freshly oxygenated water to flow from the ambient tank through the chamber and back out to the tank. Both the flush and recirculation pumps were turned on during the flush period to allow water to flow in a non-countering manner for optimally efficient flushing (Svendsen et al., 2016). When the flush pump was not turned on, the recirculation pump allowed water to only

flow through the chamber, past the oxygen probe, and back to the chamber. The wait period began when the flush pump was turned off and the recirculation pump remained on. The purpose of the wait period was to allow the enclosed water to completely circulate through the chamber before the measurement began. Following the two-minute wait, the linear decrease in oxygen consumption was measured for 10 minutes and followed by a flush to begin the next period. This protocol was implemented for all fish in all treatments.

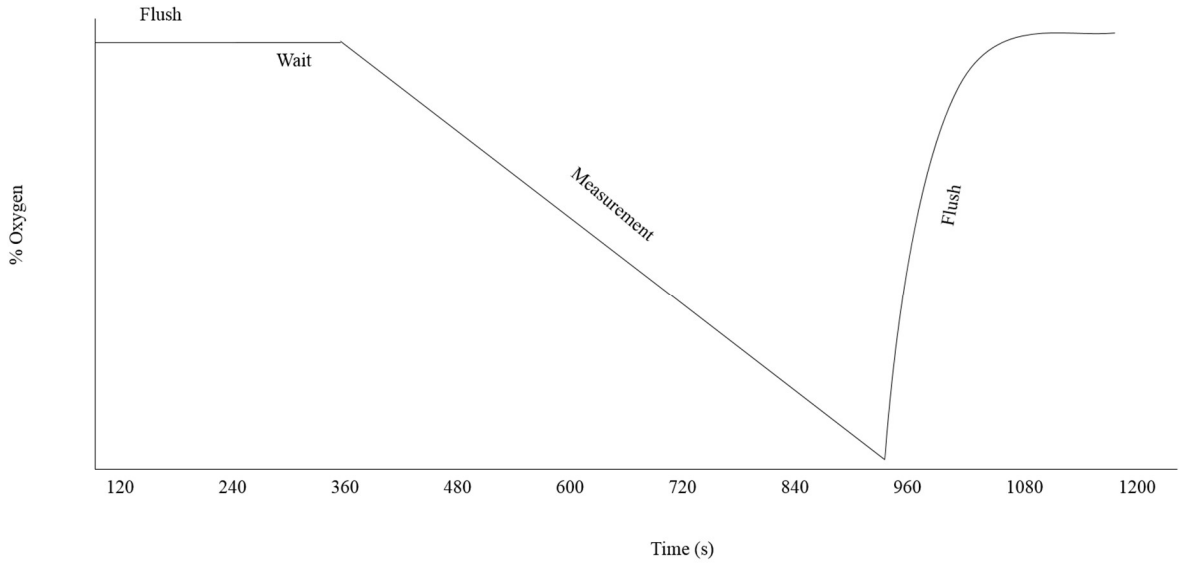

Figure 6. A measurement period began after the previous flush and was followed by a two-minute wait, a ten-minute measurement, and a four-minute flush. Oxygen levels begin at near saturation and decrease linearly during the measurement period. Figure adapted from Svendsen et al. 2016.

Background measurements were recorded without fish after every fourth set of measurements to account for potential oxygen consumption by bacteria within the system. These measurements were performed as described above, except the measurement period was 60 minutes instead of 10.

Experimental Exposure

Measurements of baseline resting metabolic rate (RMR) were obtained for each of the 24 fish under ambient conditions (16 ppt salinity; pH 6.8) and then 1 and 7 days post exposure to the experimental treatment. Following baseline RMR measurements, fish were held two per 75 L tank for 3 days in ambient water to recover from any potential handling/confinement stress. Following the 3-day hold, a 100% water change was performed to replace holding water with the assigned experimental condition (ambient, low pH, low salinity, or multi-stressor). Oxygen consumption rates were measured in individual fish 1 and 7 days after this water change.

Calculation of Oxygen Consumption Rate

Oxygen consumption rate for each fish was calculated using the following equation from Rosewarne et al. 2016:

$$y_{\text{corrected}} = [\beta (K_1 V_1 - K_2 V_2)] M^{-1}$$

where $y_{\text{corrected}}$ is fish MO2 corrected for background respiration, β is the solubility of oxygen in water at the experimental water temperature and salinity (mgO2 l^{-1} kPa^{-1}), K_1 and K_2 are the rates of decline (kPa h^{-1}) in oxygen content over time in the respirometer during the measurement phase when the animal is present and absent, respectively, V_1 and V_2 are the respirometer volumes (l) when the animal is present and absent, respectively, and M is the body mass of the animal (kg) (Rosewarne et al., 2016).

Statistical Analysis

Values are expressed as means ± standard error. Overall treatment effects on whole-body cortisol concentration and oxygen consumption rates were analyzed using repeated measures analysis of

variance (ANOVA) followed by *a posteriori* multiple comparisons (Tukey's HSD). A repeated measures ANOVA was also performed for oxygen consumption rates within day and within treatment, followed by *a posteriori* multiple comparisons (Tukey's HSD).

RESULTS

Experiment 1: Analysis of Cortisol Response

F. heteroclitus held under ambient conditions (16 ppt salinity and 4.5 pH) had whole-body cortisol concentrations of 4.2 ± 1.4 ng g^{-1} on day 1, which is in the range reported for zebrafish under control conditions (Ramsay et al., 2009). Whole-body cortisol concentration in *F. heteroclitus* did not change in response to altered salinity ($p = 0.85$), pH ($p = 0.72$), or the combination of salinity and pH ($p = 0.44$) (Figure 7). There were also no interactions between day and treatment that effected cortisol concentrations in the fish ($p = 0.06$ day X pH; $p = 0.7$ day X salinity). However, overall cortisol levels were lower on day 7 compared to day 1 and day 5 ($p = 0.0001$).

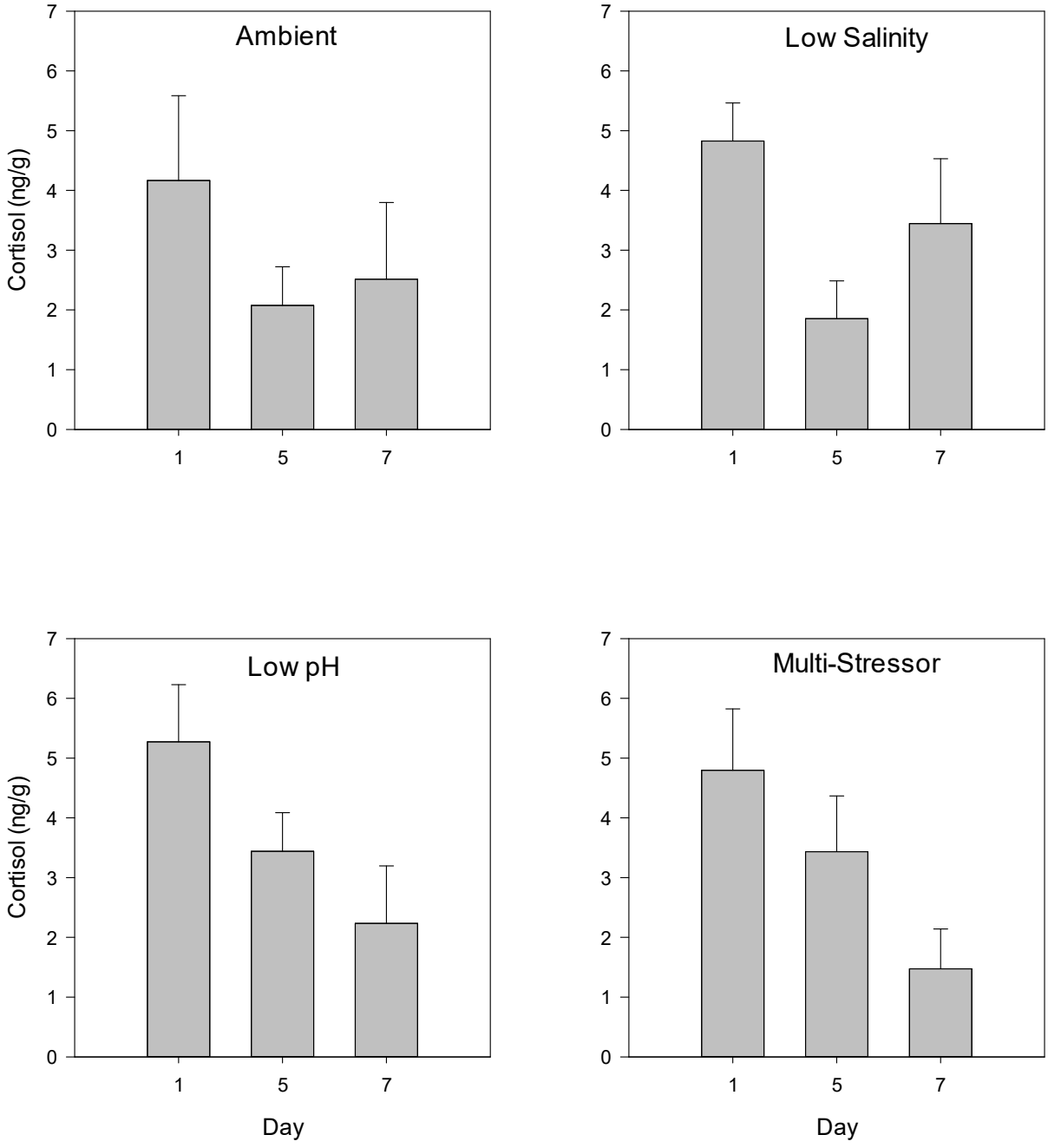

Figure 7. Average whole-body cortisol concentrations (ng g^{-1}) in *F. heteroclitus* measured 1, 5 and 7 days after transfer to experimental exposures. Ambient (16 ppt, pH 6.8), low salinity (16 ppt, pH 4.5), low pH (16 ppt, 4.5 pH), or multi-stressor (2 ppt, 4.5 pH) conditions. There was no significant effect of any treatments on whole-body cortisol concentrations, only an overall decrease over time. Data presented as mean ± SE ($p \leq 0.05$, n = 6 for all time points within treatments).

Experiment 2: Analysis of Oxygen Consumption Rate with Intermittent-flow Respirometry

The average rate of oxygen consumption for all fish prior to treatment exposure was 242 ± 153 mg O_2 kg^{-1} hr^{-1}, which is in the range of values reported by a similar study on *F. heteroclitus* (Kidder et al., 2006). Exposure to either ambient or low salinity had no effect on the oxygen consumption rate of *F. heteroclitus* (p = 0.2090 overall; 0.5525 day 1; 0.0958 day 7). However, exposure to low pH increased the fish's oxygen consumption rate (p = 0.0017 overall; 0.0127 day 1; 0.0001 day 7) (Figure 8). There were no interactive effects of salinity and pH on the oxygen consumption rate of the fish (p = 0.7476 overall; p = 0.5724 day 1; p = 0.2511 day 7). Therefore, the effect of the multi-stressor treatment on fish's oxygen consumption rate was additive. Time did not affect the fish's oxygen consumption rate within the ambient treatment (p = 0.2544) or the low salinity treatment (p = 0.3904) (Figure 9). However, fish's oxygen consumption rate increased when exposed to the low pH treatment by day 7 (p = 0.0033*) and in the multi-stressor treatment by day 7 (p = 0.0005).

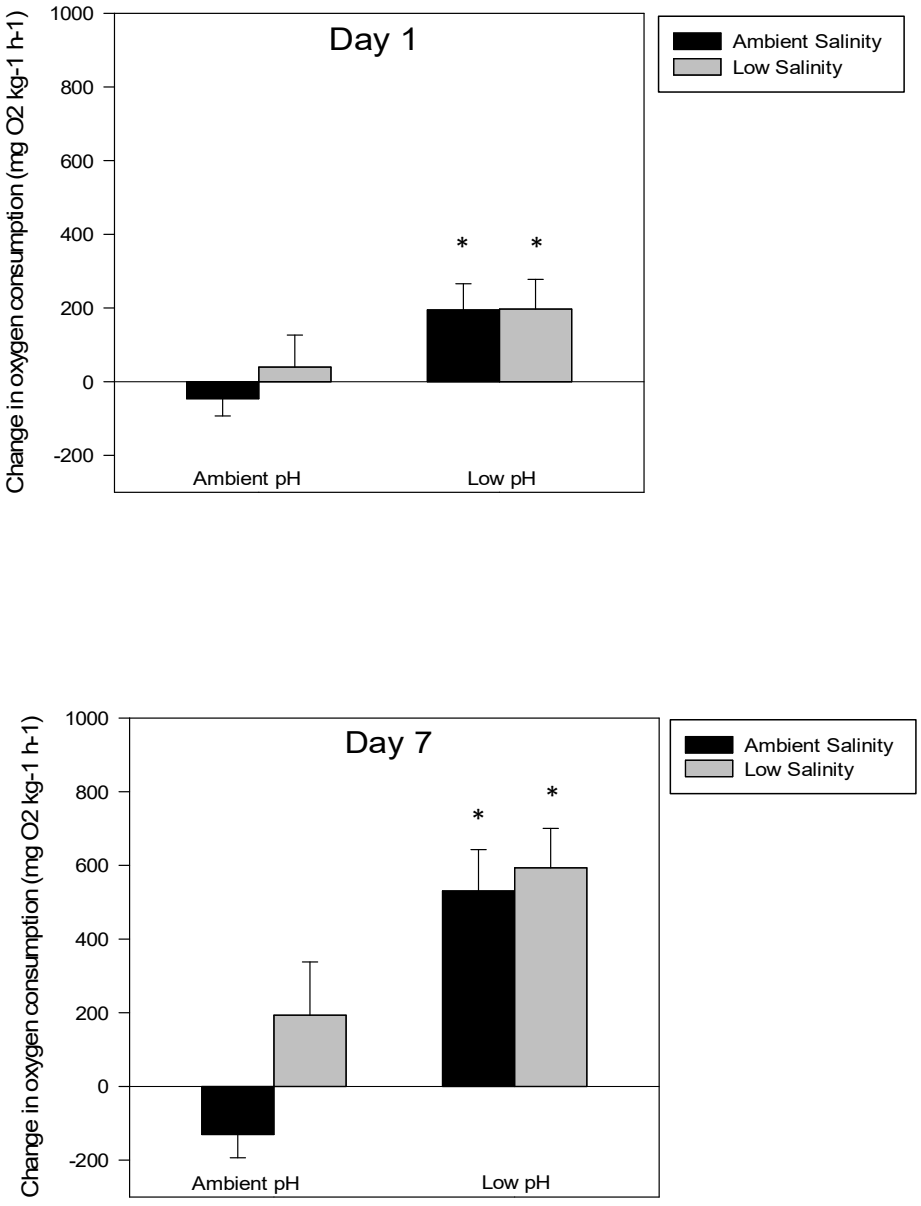

Figure 8. Average change in oxygen consumption rates from initial baseline values (mg O_2 kg^{-1} h^{-1}) in *F. heteroclitus* exposed to ambient, low salinity, low pH, or multi-stressor conditions. Day 1 and 7 represents the time post treatment exposure. Data presented as mean ± SE (n=6) (* $p \leq 0.05$, n = 6 for all time points within treatments).

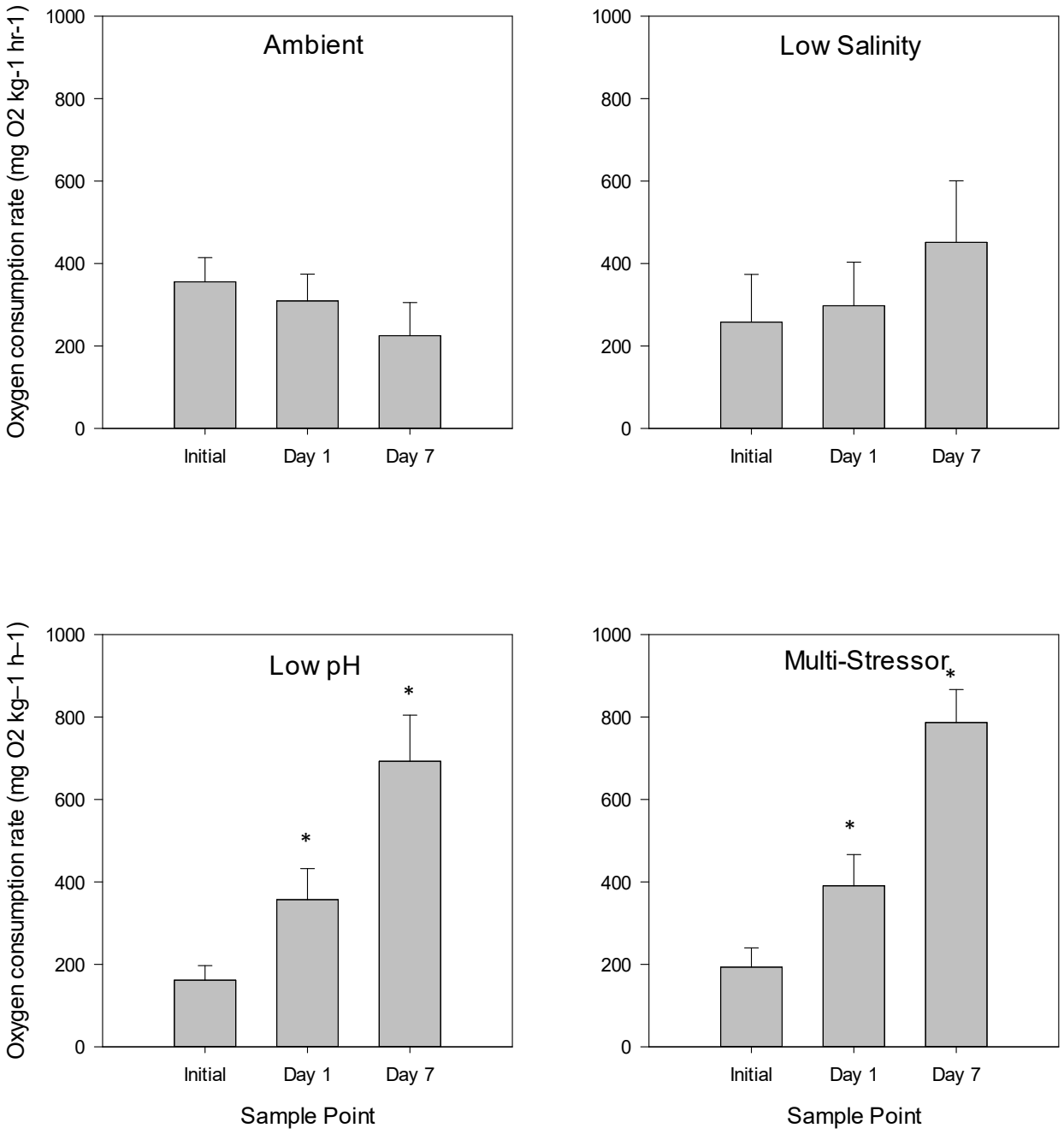

Figure 9. Average oxygen consumption rates (mg O_2 kg^{-1} hr^{-1}) in *F. heteroclitus* measured 1 and 7 days after transfer to experimental exposures. Ambient (16 ppt, pH 6.8), low salinity (16 ppt, pH 4.5), low pH (16 ppt, 4.5 pH), or multi-stressor (2 ppt, 4.5 pH) conditions. Oxygen consumption rate increased on day 7 in the low pH and multi-stressor treatments (p = 0.0033, 0.0005). Data presented as mean ± SE ($p \leq 0.05$, n = 6 for all time points within treatments).

DISCUSSION

This study provides evidence that exposure to low salinity and pH, both individually and in combination, did not result in recruitment of the primary stress response (cortisol). However, chronic exposure to low pH did result in an increase in oxygen consumption, representing an increased metabolic cost to the organism. There was not a synergistic effect of low salinity and low pH as predicted, instead the combination of the two stressors had an additive effect on RMR driven mainly by the decrease in environmental pH. As well, the continual increase in RMR with time (highest rates at day 7) suggests that extended exposure to low pH may become detrimental for *F. heteroclitus* if it is not able to acclimatize to offset the increase in energetic costs of living in a low pH environment.

Multi-stressor effects of altered salinity and pH on whole-body cortisol

In this study I hypothesized that the combined effects of low salinity and low pH would result in the recruitment of the primary stress response (shown by increased whole-body cortisol levels) in a synergistic fashion. The release of cortisol during the primary stress response triggers the activation of homeostatic mechanisms which aid in acclimation to salinity and pH in *F. heteroclitus* by enacting ion pumps and secretion pathways in mitochondria-rich gill epithelial cells (Bern and Madsen 1992; Jacob and Taylor 1983; Lin and Randall 1993; Mancera and McCormick 1998; Singer et al., 1998; Tipsmark et al., 2002). In *F. heteroclitus*, cortisol peaks as early as 10 minutes post salinity transfer and may remain elevated or begin to decrease depending on the intensity of the stressor (Jacob and Taylor 1983; Marshall et al., 1999; Morgan et al., 1997; Tsui et al., 2012). Changes in environmental pH have also been documented to trigger the primary stress response with acute transfer to a pH of 4.5 causing circulating levels of cortisol to increase

up to 500% of baseline in rainbow trout and carp (Kakizawa et al., 1996; Nagae et al.). These levels remained elevated for up to two days post exposure indicating a high level of stress exhibited by the fishes (Kakizawa et al., 1996; Nagae et al., 2001) suggesting exposure to low pH is a substantial stressor. Despite the strong evidence in the literature for cortisol's role in initiating homeostatic mechanisms for ion regulation and acid-base balance, exposure to low salinity and/or low pH did not result in a significant increase in cortisol in the *F. heteroclitus* used in this study at either 1 or 7 days post transfer. Mitochondria-rich cells are more prevalent in *F. heteroclitus* than most euryhaline teleosts enabling these fish to rapidly alter ion secretion and absorption rates resulting in quick acclimation to fluctuations in salinity and pH (Marshall et al., 1999; Tipsmark et al., 2002). It is possible in our fish cortisol peaked immediately post transfer and had returned to baseline levels by the first sample point on Day 1. This return of cortisol to baseline levels suggests the necessary homeostatic regulation mechanisms, such as expression of transport proteins and chloride channels, have been enacted (Hu et al., 2016). Mechanisms, such as sodium potassium pumps and sodium hydrogen exchangers, either directly or indirectly utilize ATP to facilitate the regulation of ion and water balance (Scott et al., 2004a). Although *F. heteroclitus* are able to produce and utilize transport proteins quickly when needed, chronic exposure to a low salinity and/or low pH environment would require continual maintenance of ion and proton levels creating an increased energetic demand which would in turn increase the oxygen consumption/RMR of the fish.

Multi-stressor effects of salinity and low pH on resting metabolic rate

Measurements of oxygen consumption in *F. heteroclitus* demonstrated that fluctuations in salinity did not result in an increase energetic costs; whereas a decrease in environmental pH, either alone

or in combination with low salinity, resulted in an increase in the RMR. RMR of the fish exposed to low pH continued to increase with time with highest oxygen consumption rates at day 7.

Although it has been shown that the southern population exhibits a behavioral preference for brackish water, they are still extremely well adapted to rapid changes in salinity represented by their ability to maintain normal levels plasma ion concentration and Cl^- secretion rates by 2 days after exposure to altered salinity (Bucking et al., 2012). These fish experience naturally altered salinity with tidal, seasonal, and weather events which may result in increased metabolic rates for short periods of time when above normal ion regulation is needed. Local adaptation is very strong in *F. heteroclitus* and only rarely experienced environments act as stressors (Schulte 2014). The fish used in this study were captured from a tidally influenced stream which experienced salinity fluctuation up to 10 ppt. However, pH in the stream remained stable at 6.7 to 6.9. Therefore, it is possible that the specific population used in this study may be better adapted to salinity change than pH change.

While the fish used in this study were from a stream with close to neutral pH, *F. heteroclitus* have been found living in blackwater type environments with pH levels similar to those tested in this study (4.5), however pH levels do not fluctuate with these events as much as salinity does. Events that cause rapid alterations to pH are rare, such as acid-rain or drastic increase of CO_2. Therefore, in these cases the fish must expend energy above baseline rates to acclimate to this stressor

Increased RMR represents an increase in the energetic cost of *F. heteroclitus* acclimating to low pH. Energy, in the form of ATP, is required for fishes to regulate acid-base concentration by

altering net transport of acidic or basic ions across the gills (Claiborne et al., 2002). Exposure to low pH also requires fishes to increase acid concentration via either electroneutral Na^+/H^+ exchanger (NHE) (Patrick and Wood 1999) or vacuolar H^+-ATPase electrically linked to a Na^+ channel (Lin and Randall 1993). The H^+-ATPase model is widely accepted in freshwater fishes; however, the NHE model is observed in most marine and some euryhaline fishes, including *F. heteroclitus* (Choe et al., 2002; Claiborne et al., 1999; Wood and Marshall 1994). Therefore *F. heteroclitus* reduce Na^+ excretion while increasing Cl^- excretion resulting in a net acid loss without altering influx rates of either ion. However, in multi-stressor environments the ability to take up Na^+ can become inhibited by low pH (Patrick and Wood 1999).

Within the low pH streams on the island, water levels changed rapidly seasonally and were non-existent during the summer months. This suggest that the fish living there are able to take refuge elsewhere when the streams are dry, but take advantage the streams following rain events. This may indicate that there is an advantage in some cases to incur the energetic costs of living at low pH, possibly to access food or refuge from predators that is not normally available. However, if RMR were to continue to increase due to low pH exposure at the rate observed in this study, it is possible that the fish would exhibit negative effects on growth, feeding, and reproduction which would lower the fish's fitness (Barton 2002).

Future directions & conclusions

The goal of this study was to analyze cortisol concentration as a primary stress response and RMR as a measure of secondary stress response to determine the effects of altered salinity and/or pH on the southern subspecies of *F. heteroclitus.* In summary, the results suggest that cortisol

concentration is unaffected by exposure to low salinity and/or low pH. However, the RMR of *F. heteroclitus* increases with time due to exposure of low pH. Increased oxygen consumption is most likely a direct result of the elevated ATP demand needed to increase ion transport and regulation to maintain acid/base balance. In conclusion, the results of this study suggest that southern *F. heteroclitus* are physiologically well equipped to cope with fluctuations in environmental salinity; but reductions in low pH, either alone or in combination with varying salinity, may place increased energetic demands on the organism that may ultimately impact its fitness. The physiology of organisms living in multi-stressor environments as well as the interacting effects of those stressors remain a valuable area of study and provides many opportunities for future research.

The next step to fully understanding the implications of varying salinity & pH on *F. heteroclitus* is to investigate the physiological and cellular mechanisms underpinning the increase in resting metabolic rate. Specifically, ionic and acid-base regulation in *F. heteroclitus* will be an important area of research and measurement of the expression level and activity of the major ion transport proteins will provide a more in-depth view of what physiological processes are requiring energy to acclimate to altered salinity and pH. Some examples are Na^+-K^+-ATPase, NKCC1, and CFTR genes which regulate ion flux rates by altering transport proteins and cause cell swelling to increase absorption in low salinity and pH environments (See Appendix 2).

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Appendix One:

Statistical Tables

Experiment 1: Whole-Body Cortisol Analysis			
	DF	F Ratio	Prob > F
Day	2	10.5745	0.0001*
pH	1	0.1342	0.7236
Salinity	1	0.0380	0.8504
Day X Salinity	2	3.0197	0.0575
Day X pH	2	0.3599	0.6995
Salinity X pH	1	0.6527	0.4425
Day X Salinity X pH	2	0.5948	0.5554

Experiment 2: Oxygen Consumption Analysis			
	DF	F Ratio	Prob > F
Day	2	14.8779	<.0001*
pH	1	3.4337	0.0787
Salinity	1	0.5590	0.4634
Day X Salinity	2	1.6652	0.2020
Day X pH	2	11.6671	0.0001*
Salinity X pH	1	0.0135	0.9085
Day X Salinity X pH	2	0.7229	0.4916

Experiment 2: Difference in Oxygen Consumption from Initial to Day 1			
	DF	F Ratio	Prob > F
Salinity	1	0.3650	0.5525
pH	1	7.0127	0.0127*
Salinity X pH	1	0.3294	0.5724

Experiment 2: Difference in Oxygen Consumption from Initial to Day 7			
	DF	F Ratio	Prob > F
Salinity	1	3.0548	0.0958
pH	1	23.0440	0.0001*
Salinity X pH	1	1.3969	0.2511

Appendix Two

Genetic Expression Analysis

Preparation of RNA & Genomic DNA from Gill Tissues

Gill tissues from *F. heteroclitus* used for whole body cortisol analysis (experiment 1) were kept frozen with liquid nitrogen while powdered with mortar and pestle. 50-100 mg of powdered tissue was homogenized using a PowerGen 125 electric homogenizer (Fisher Scientific, Waltham, MA, USA) in 1 ml TRIzol Reagent (LifeTechnologies California, USA). The electric homogenizer was cleaned between samples with ethanol and deionized water. Resulting homogenates were used for RNA extraction following the TRIzol manufacturer's protocol with modifications. Gill homogenates were centrifuged at 12,000 *g* for 10 minutes at 4°C and supernatant was transferred to a new 1.5 mL Eppendorf tube and incubated at room temperature for 5 minutes. 200 µl of chloroform was then added to each sample and hand inverted for 5 minutes. Samples were centrifuged again at 12,000 *g* for 15 minutes at 4°C and supernatant was transferred to a new 1.5 mL Eppendorf tube. 400 µl of 100% isopropanol was added to each sample and incubated for 10 minutes at room temperature. Samples were again centrifuged at 12,000 *g* for 10 minutes at 4°C and the supernatant was discarded, leaving only the RNA pellet. The pellet was washed with 1 mL of 75% ethanol to improve purity. The pellet and wash were centrifuged at 7,500 *g* for 5 minutes at 4°C, wash was discarded, and pellet was air dried for 5-10 minutes. Once dried, the pellet was re-suspended in 20-50 µl RNase-free H_2O and incubated in a heat block for 10 minutes at 57-59°C. RNA concentration was measured with a NanoDrop UV spectrophotometer (NanoDrop Technologies). All total RNA samples were then stored at -80°C until cDNA synthesis.

cDNA synthesis

First strand complimentary DNA (cDNA) was synthesized from 2µg of total RNA in a 20 µl reaction using random primers [200 ng (Invitrogen)] and RevertAid H^- V Reverse transcriptase [200 $U\mu l^{-1}$ (Fisher Scientific)] with the manufacturer's 5X reaction buffer and RNase OUT [40 μl^{-1} (Invitrogen)] at 42°C for 60 minutes. Negative control reverse transcriptase (NRT) and no-template control (NTC) reactions were ran with each set of RNA samples. cDNA was diluted in nuclease-free H_2O to a final volume of 50 µl. Samples were stored at -20°C until further analysis.

Primer design/sources and testing

Target gene expression was analyzed using primers from published literature (Table A). Primers for the genes of interest and housekeeping gene were tested using real-time PCR (polymerase chain reaction) consisting of 0.5 µl pooled cDNA (from a randomly selected subset of the samples), 0.1 µl Platinum®Taq (Life Technologies, Grand Island, NY), and 10 µM of forward and reverse primers. Each PCR was duplicated and consisted of 38 cycles which included 30 seconds at 94°C, 30 seconds at 72°C, and reaction ended with a final extension of 15 minutes 72°C (Scott et al., 2004a). A negative control was added to each PCR set that contained all reaction components with the exception that DEPC-treated nuclease-free water (Life Technologies, Grand Island, NY) instead of a cDNA template. PCR products were verified for the correct base pair size with by electrophoresis on ethidium bromide stained 1% agarose gel alongside a 1 Kbp ladder (Fisher Scientific). Primer sets that are successful in producing a single product the appropriate size (50-200 bp) will be utilized for gene expression analysis with QRT-PCR.

Table A. Primers used for qRT-PCR of ion transport genes (Scott et al., 2004a).

Gene	**Primer (5'-3')**	**Accession Number**
Na^+-K^+-ATPase-α_{1a}	F: AAG ATC ATG GAG TCC TTT AAG AAT CTG	AY057072
	R: CAC CTC CTC TGC ATT GAT GCT	
CFTR	F: AAT CGA GCA GTT CCC AGA CAA G	AF000271
	R: AGC TGT TTG TGC CCA TTG C	
NKCC1	F: CCC GCA GCC ACT GGT ATT	AY533706
	R: GCC ATC TGT GGG TCA GCA A	
EF1α	F: GGG AAA GGG CTC CTT CAA GT	AY430091
	R: ACG CTC GGC CTT CAG CTT	