DISSERTATION

ECO-PHYSIOLOGICAL DRIVERS OF GEOGRAPHIC RANGE LIMITS IN TWO CLOSELY RELATED EURYHALINE FISH SPECIES

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2021

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ABSTRACT

ECO-PHYSIOLOGICAL DRIVERS OF GEOGRAPHIC RANGE LIMITS IN TWO CLOSELY RELATED EURYHALINE FISH SPECIES

A fundamental goal in evolutionary ecology is to understand the problem of why species occur in some environments and not others. Indeed, a general pattern in nature is that many organisms occupy only a subset of the total range of the environments they are physiologically capable of tolerating. Theory suggests that the abiotic environment can constrain the distributions of species, but testing the relative roles of different mechanisms in shaping species distributions has proven to be a major challenge for both plants and animals. In fish, salinity tolerance is a defining factor in shaping the ranges of many species. Nonetheless, the influence of salinity tolerance on patterns of dispersal and local adaptation are understudied for most species. Euryhaline fishes are capable of acclimating to a wide range of salinities, yet may exhibit a preference for a particular salinity. For example, previous work in euryhaline teleosts indicates that crossing a salinity gradient typically results in increased oxygen uptake, energetic costs, and activation of the stress response. Thus, plastic or evolved tolerance to increased salinity might come at the expense of fitness-related traits (i.e., locomotion, feeding, mate acquisition, etc.), but few studies have investigated the potential for such trade-offs. Maintaining ionic and osmotic homeostasis in the face of a salinity change is critical for survival, but also energetically costly. Efficient osmoregulation relies primarily on the gills, but the process is complicated given that freshwater and saltwater fishes differ in the direction of ion transport through the gill epithelia. Thus, proper restructuring of the gills is fundamental to surviving a salinity transition. This plastic response has been observed in

euryhaline fishes, however there is intra- and interspecific variation in the timescale of this process. The endocrine system plays a significant role in salinity acclimation, and in euryhaline teleosts salinity exposure increases the concentration of circulating plasma cortisol to facilitate osmoregulation. Previous work indicates that cortisol is involved in promoting structural changes in both fresh and saltwater gills, but its role in osmoregulation and adaptation differs between the two types. Thus, comparisons of cortisol concentrations can provide insight into the roles of local adaptation and plasticity for euryhaline fishes that exist along a salinity gradient. On the island of Trinidad, the euryhaline guppy (*Poecilia reticulata*) is confined to freshwater whereas the closely related swamp guppy (*Poecilia picta*), co-exists with the guppy in freshwater, but also spans into brackish and saltwater. To understand this pattern, we employed an integrative approach to investigate the mechanisms and potential trade-offs that may exist upon exposure to increased ambient salinity as a result of seasonal and daily tidal fluctuations. We examined the effect of a gradual salinity increase on sustained swimming (U_{CRIT}) for *P. reticulata* and burst swim performance for *P. reticulata* and *P. picta* by estimating salinity performance curves (SPCs) using field collected fish. We mimicked the same salinity challenge in the lab and measured the ability of lab reared *P. reticulata* and *P. picta* to maintain internal osmolality. In addition, we used a novel method to quantify differences in circulating cortisol levels in *P. picta* allowing us to infer whether populations along the salinity gradient track stable versus variable salinity levels by adjusting their cortisol levels. Our experiments revealed that *P. reticulata* can maintain sustained swimming performance across a broad range of salinities and achieves peak performance at the isosmotic point, confirming its euryhaline ability. In contrast, both *P. reticulata* and *P. picta* initially experience a drop in burst swimming performance when exposed to salinity challenge, but are able to acclimate over time to higher salinities and re-establish their performance. However, this

acclimation response occurs much more quickly in *P. picta* compared to *P. reticulata*. The slower acclimation response of *P. reticulata* could potentially make them more vulnerable to predation risk when they attempt to become established in brackish water, thus contributing to their ranges being restricted to freshwater in Trinidad. Cortisol analyses along the salinity gradient provide support for *P. picta* having the ability to plastically increase circulating cortisol levels in response to daily fluctuations in salinity.

Overall, these results demonstrate how understanding the physiological responses to salinity can inform which mechanisms do and do not contribute to distribution patterns in nature.

ACKNOWLEDGMENTS

There are many individuals that have played a significant role in this journey. I would first like to thank my advisor, Cameron Ghalambor, and co-advisor, Lisa Angeloni, for their constant guidance and support. Dealing with the ups and downs of a graduate student advisee is surely not an easy task, so I would like to both thank you for being such incredible mentors and apologize for everything you had to put up with over the last six years. This dissertation would not be possible without funding from an NSF grant awarded to Cameron as well as the opportunity to conduct fieldwork while on a Research Assistantship.

Cameron – I first off want to thank you for taking a chance on me, providing me with the opportunity to join your lab. You always pushed me to think in ways that I never imagined while improving my project each step of the way. You have a special gift when it comes to transforming my ambitious, and sometimes off-the-wall, research questions/ideas into an attainable, interesting, and relevant project. I will certainly miss our brainstorming sessions that could last several hours. You not only challenged me to think in new ways, but also valued my ideas and input. It was these meetings that made me feel like more of a collaborator than a graduate student.

Lisa – I want to thank you for becoming my co-advisor and making me feel welcome and valued in your lab. I am not sure you will ever understand how much you have helped me during my time in graduate school. At my lowest of lows, you refused to let me quit and pushed me to move forward, keeping my eye on the goals I set for myself. I am not sure I would have made it through the last six years without your encouraging words and advice. I came away from every interaction wanting to become a better scientist, mentor, and writer. During our meetings, you were always a fantastic sounding board, but it was your input and suggestions to improve my research

and writing that proved to be invaluable to making this work better. You are an amazing support system and advocate not only for me, but for every graduate student in the department. Your immense efforts to improve graduate student life in this department as well as your compassion and empathy certainly does not go unnoticed or unappreciated.

I would like to thank my committee, Ryan Earley and Chris Myrick. Ryan – Thank you for making me an honorary member of the Earley Lab. Your lighthearted attitude, optimism, and ability to make science fun has really made my time working at your lab in Alabama an incredible experience. I am forever grateful for all the time and effort you put into improving my dissertation. It has been a real honor and pleasure getting to work alongside you during my visits down south. Chris – Thank you so much for all your guidance and advice in my attempts to carve my own path in the field of fish physiology. I appreciate your willingness to meet with me whenever I was stuck on how best to proceed with my projects. My only regret is that my metabolism work did not pan out the way that I would have liked as it would have provided even more opportunities for the two of us to work closely together.

I certainly would not be where I am today without being provided the opportunity to conduct research as an undergraduate in Matthew Wund's lab at The College of New Jersey. He took a chance on me as one of his first undergraduate researchers and for that I am incredibly grateful. Not only did he introduce me to the world of biological research, but also sparked my passion for everything related to fish, especially stickleback. His encouragement and guidance extended way beyond my time as an undergraduate and he became one of my greatest support systems as I navigated my way through graduate school.

This dissertation would not have been possible without the incredible support from former undergraduate volunteers and honors students: Michelle Moyer, Richard Evans. Rachel Bockrath,

Kyndall Zeller, and Jovan Vincent. It was an honor and pleasure getting to work with each one of you. I want to thank you all for your help and support and for making positive contributions to this work. I enjoyed working with you to build your theses from the ground up and seeing you all work so hard to achieve your goals. I know you all will do incredible things in life, and I am looking forward to hearing all about it.

I would also like to give a huge thanks to Hammond and Nigel Noriega for their amazing hospitality throughout my several stays in the Hamgel field station in Trinidad. They provided our lab members with an incredible place to stay and conduct research. Spending long periods of time in another country is such an amazing experience, but it can also be overwhelming. Hammond and Nigel not only showed me everything their island had to offer, including introducing me to fantastic food, places, and people, but they also treated me like family. I am forever grateful for the opportunities they provided to immerse me in their culture.

Moving on to general acknowledgements beyond the dissertation, I want to thank my labmates in both the Ghalambor and Angeloni Labs, past and present: Helen, Julian, Katie, Corey, Emily, Alisha, Maybellene, Porsche, Alex, Rebecca, Dale, Brett, Chris S., Chris K., Casey, and Jennifer. You are all incredible researchers, advocates, and friends. I am grateful that our paths crossed at CSU and I would not have made it through the last six years without your support and valuable suggestions. Thank you for all your comments and feedback throughout the years. I appreciate each one of you and I am looking forward to seeing you wherever our paths may cross in the future. I wish the best for you all.

To my G-RHAF family, it has been a blast getting to know and work with each one of you. I could not imagine a better group of people to share a lab space with during my time here. You are an incredibly bright, supportive, and fun group of people and I look forward to continuing to

celebrate your amazing and well-deserved accomplishments along the way. To Chris Funk, Kristen Ruegg, and my unofficial fifth committee member, Kim Hoke, I want to say thank you for all the support and guidance over the last six years. I aspire to be a better mentor because of you.

I want to express my deepest gratitude to Jennifer Neuwald, Mark Simmons, and Colleen Webb for providing me with the opportunity to be your TA for BZ 220: Introduction to Evolution. I will always look back on my teaching experiences with such fond memories. You have all contributed to making me a better instructor and mentor. Your commitment to teaching and improving the education of your students has been incredibly inspiring. I wish I can one day be half the instructor that the three of you are.

In addition to teaching in the classroom, I was also fortunate enough to be selected to do behind the scenes curriculum development for LIFE 102 and 103 over the last two years. I want to thank Kim Hoke, Tanya Dewey, and Meena Balgopal for welcoming me into your group and allowing me to learn from you. Having the opportunity to work alongside such caring and hardworking educators has inspired me to pursue a career as an instructor. Thank you for such an incredible and fun experience.

I could not write this acknowledgements section without thanking the incredible people that comprise the Biology staff. You all do not receive enough credit and praise for the work that you do. You have saved me a countless number of times and for that I am extremely grateful. Some of my best graduate school moments were spent having impromptu conversations in the main office over some cookies I stole from the seminar snack cabinet. I am glad that I was able to get to know each one of you. You are an incredible group of people.

I must send a huge shoutout to my Fall 2015 Biology cohort and all the other friends I made throughout my time at CSU. Graduate school is filled with many ups and downs, and I could not

imagine a better group of people to share this ride. You have all made the last six years an unforgettable experience. I enjoyed every moment we were able to spend together whether we were talking about science or making pacts not to even mention it. You were the support system I never knew I needed but could not live without. It has been incredible getting to know you all both inside and outside of the lab. You have all really made graduate school worth it. I sincerely hope our paths cross again (and again) soon and I look forward to the days where we can sit around and reminisce about the good ol' days of our times at CSU.

I can honestly say that I would not be where I am today without my Princeton Ecology and Evolutionary Biology family. You have inspired me in so many ways and constantly pushed me to pursue my dreams. In the words of Henry Horn, it has been "an enlightening pleasure to know you." Through our interactions and experiences, I have become a better scientist and thinker. I have enjoyed celebrating the various successes and accomplishments you have achieved over the years, and I look forward to celebrating the many more to come. Thank you for welcoming me into your EEB family whether I am in New Jersey, Colorado, or elsewhere.

To my friends from my hometown of South River, NJ and The College of New Jersey – I cannot thank you enough for putting up with me from afar this entire time I have been in Colorado. It has been rough being so far away, but please know that I very much appreciated the various calls and texts over the years. You were all an incredible support system, but I am even more grateful that you were never afraid to put me in my place when I needed it. I am so fortunate to have friends like you in my life and I could not imagine what this journey would have been like without you. I credit each one of you for getting me to where I am today.

Last, but certainly not least, I want to give a special thank you my family. Mom, Dad, Scott, David, Krystal, Kaysie, Drew, Hunter, Bryce, and Collyns – I know these past six years have not

been smooth sailing. I wish I was able to visit more, but now that this chapter is coming to an end, I am going to make every effort I can to make up for lost time. Thank you for putting up with me, allowing me to vent all my frustrations, and for your constant love and support. I could not have done this without you.

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CHAPTER 1: INTRODUCTION

The range limit of a given species is the geographic space that it occupies in nature (Sexton et al. 2009). Surprisingly, given the vast areas of land and bodies of water, many species tend to be confined to relatively few regions regardless of opportunities for expansion (Gaston 2003). Describing the structural dynamics of a species' range (i.e. shape, size, location, boundaries, etc.) (Brown et al. 1996) and identifying which factors play a role in shaping range limits (Lawton, et al. 1994; Brown et al. 1996) are fundamental goals of biologists attempting to understand the factors that control distribution patterns. Given that environments and landscapes can experience both gradual and dramatic transformations over time, much attention has been focused on predicting how species will respond to such changes (Davis and Shaw 2001; Brown et al. 1996; Gaston 2009; Sexton et al. 2009). This leads to a fundamental question in eco-physiology: ***Why do species inhabit some environments and not others?***

One conceptual framework that has been used to understand species distributions is the contrast between the fundamental and realized niche. The fundamental niche relates to the capacity of a species to theoretically exist in a given area provided that the conditions are favorable to promote survival, reproduction, and overall positive population growth (Chase and Leibold 2003). However, organisms only naturally exist in environments where they can physiologically survive and are successful in competing to acquire resources and evading predation, comprising their realized niche (Pulliam 2000). Previous research indicates that both abiotic and biotic factors play a role in determining how species are distributed in their realized niche. However, the relative importance of each of these factors can differ between species and even between populations of the same species (Dunson and Travis 1991; Holt 2003; Sexton et al. 2009). For example, species boundaries can often arise as a result of habitat heterogeneity wherein barriers, such as a mountain

range or large bodies of water, prevent species expansion. Some factors that determine boundaries may not be as dramatic, including gradients of temperature (Perry et al. 2005; Chen et al. 2011), precipitation (Kirkpatrick and Barton 1997), and salinity for aquatic organisms (Nejrup and Pedersen 2012; Torres-Dowdall et al. 2013). Antagonistic biotic interactions, such as predation, competition, parasitism, and even plant-animal relationships can be essential to defining the geographic distributions, and thus realized niche of a species (Hargreaves et al. 2014). However, a species range is often delineated by interactions between biotic and abiotic factors (Sexton et al. 2009; Coulson et al. 2011). For example, Connell's (1961) classic experiment on distribution patterns of two barnacle species revealed that abiotic (tidal fluctuations) and biotic (competition) interactions jointly shaped their ranges. Thus, understanding how organisms respond to the joint effects of abiotic and biotic factors is critical to understanding species distributions.

Few ecological studies have attempted to elucidate the integrative relationship between biotic and abiotic factors in determining community dynamics, including both inter- and intraspecific competitive interactions (Dunson and Travis 1991). In fact, emphasis on abiotic factors versus biotic factors in determining dispersal patterns and community membership has shifted throughout the history of ecology. Grinnell's (1917) description of the niche incorporated both biotic and abiotic influences, however, his work on the California thrasher (*Toxostoma redivivum*) predominantly focused on the role of abiotic factors in shaping the species' range. In addition, Hutchinson's "Concluding Remarks," (1957) also placed particular emphasis on biotic factors when providing the distinction between fundamental and realized niches. More recently, emphasis has been placed on the role of biotic factors in determining species assemblage as a complement to the role of abiotic factors (Jackson and Harvey 1989; Tonn 1990).

Previous assessments of geographic range limits reveal that species' boundaries are not static, but rather fluctuate over time (MacArthur 1972; Holt 2003). When investigating shifts in a species' range, much emphasis is placed on changes in their immediate environmental conditions; however, evolutionary dynamics within a population can contribute to expansions/contractions allowing for individuals to be able to tolerate novel conditions or become intolerant of current conditions (Kirkpatrick and Barton 1997). Causes of species' expansion/contraction can occur over short or long timescales. Rapid range shifts can involve changes that occur in the environment, including anthropogenic effects. For example, removal of physical barriers previously preventing a species from expanding or the migration of new competitors into the environment can create an increase or decrease in dispersal events (Holt 2003). Gradual changes in geographic range limits can also involve evolutionary mechanisms, without explicit environmental changes. Such fluctuations are spearheaded by evolutionary changes in the traits of a particular species which can often occur without directional pressures from the external environment (Holt 2003). Therefore, accumulation of trait variations, such as those creating physiological diversity, can lead to the evolution of species' ranges (Bozinovic et al. 2011). An adaptive trait can thus flourish in an environment, leading to a shift in the geographic distribution of a species. In other instances, changes in range limits may rely on the process of local adaptation (Holt 2003).

Selective pressures due to habitat heterogeneity can promote local adaptation to specific environments within a species (Mazer and Damuth 2001). Local adaptation is evident when a population exhibits higher levels of fitness in their native habitat versus their non-native habitat (Kawecki and Ebert 2004). Population differentiation can allow for locally adaptive phenotypes to be both generated and maintained (Skúlason et al. 1999; Schluter 2000). However, gene flow between conspecific populations can lead to homogenization, thus inhibiting adaptive responses

to local conditions and constraining local adaptation (Sunnucks 2000; Kawecki and Ebert 2004). For isolated populations or populations where gene flow is not occurring between heterogeneous environments, local adaptation to specific conditions, causing limited tolerance to specific environmental factors, can reduce the capacity of a species or population to expand beyond their current geographic range (Mott 2010). In the event where a species migrates beyond their current range, any reduction in fitness or performance can have detrimental effects on a species' chance of survival as well as reduce the likelihood of colonization events into novel habitats.

Phenotypic plasticity plays an important role in allowing species to colonize novel environments (West-Eberhard 2003) and thus shapes patterns of evolution following an introduction to a new habitat (Pfennig et al. 2010). Plasticity itself can evolve, and selection on plastic traits can lead to changes in the level of plasticity, including increases or decreases in the level of plasticity (Baldwin effect) or canalization of a particular phenotype (i.e., genetic assimilation) (Crispo 2007; Pigliucci 2006). In the event where plasticity in a trait is maladaptive, selection can lead to reductions in plasticity for that particular phenotype (Crispo 2007; Ghalambor et al. 2015). Thus, exhibiting plastic responses in interacting traits can promote (or decrease) survival in the face of changes an organism might encounter in their current habitat or variations experienced when migrating to a novel environment. Acclimation ability is a type of plasticity that involves biochemical, cellular, and other reversible physiological changes that occur in response to exposure to an environment on the timescale of days to months (Huey and Berrigan 1996; Huey et al. 1999). Because acclimation typically results in adaptive changes that increase performance and fitness, acclimation ability may play an important role in shaping patterns of dispersal, range expansions, and geographic range limits. However, the timescale at which environments change

versus the timescale at which acclimation occurs may be a critical factor in determining whether individuals are able to establish themselves and form sustainable populations.

Species distributions are rather complex in nature and most constraints to dispersal are rooted in the physiological limitations of an organism in response to ambient environmental conditions (Pörtner and Peck 2010; Seebacher and Franklin 2011). Therefore, when attempting to determine dispersal potential for a given species in a novel environment, it is important to identify and quantify specific traits indicative of physiological performance for a new suite of abiotic conditions representative of the new environment (Behrens et al. 2017). It is well documented that the abiotic factors that an organism experiences in its environment can place restrictions on habitat expansion. For example, climate conditions, including temperature, play a significant role in limiting the range of environments that a species can occupy (Jackson et al. 2001). The internal temperatures of ectothermic species are directly influenced by their external environment; thus temperature can influence patterns of dispersal at both large and small geographic scales (Grossman and Freeman 1987). However, in areas where temperature remains stable throughout the year, such as in the tropics, other abiotic factors, such as salinity, can play a more significant role (Castillo et al. 1996).

Influence of Salinity on Aquatic Species

Among the many abiotic factors that organisms must endure in their natural habitat, tolerance to salinity is one of the most widely studied in fishes (Shikano and Fujio 1997; Shikano et al. 2001; Lisboa et al. 2015). The majority of teleost fish species (~95%) are incapable of acclimating to salinity changes and are considered to be stenohaline while the remaining ~5% are euryhaline and can thus tolerate salinity fluctuations (McCormick 2011). Since crossing along a salinity gradient typically results in a rapid osmoregulatory challenge, the freshwater-saltwater interface can act as

a physical barrier delineating the range limits of some euryhaline species. Maintaining consistent internal ion concentrations (~300 mOsmol/L for teleosts) is essential for survival in osmoregulating fishes. Thus, fishes living in freshwater face the challenge of taking in Na^+ and Cl^- ions through their gill epithelium from their relatively dilute surrounding as well as minimizing ionic loss in order to maintain ion homeostasis (Kumai and Perry 2012). Saltwater species have adapted to their high salinity environments by evolving mechanisms that allow them to replace water lost through the process of osmosis and also remove the large concentrations of salt ions that they take in from their surroundings (Wurts 1998) (see Figure 1.1).

Plasticity plays an important role for acclimation and colonization events in fish species that invade freshwater habitats from ancestrally marine populations and vice versa (Lee and Bell 1999). Truly euryhaline species must be able to regulate internal plasma ions when faced with changes in salinity whether these changes are rapid (e.g., flooding events or short-term migrations) or slow (e.g., gradual, seasonal changes) (McCormick 2011). The ability to transition between freshwater and saltwater environments relies on plasticity of tissues essential to osmoregulation, such as the gill epithelium (Evans et al. 2005). There are several metabolic changes that also occur during acclimation to hyper- and hypo-osmotic conditions (Soengas et al. 2007). Gills, in addition to their respiratory function, aid in excretory and osmoregulatory processes (Sabóia-Moraes et al. 2011). Adaptative plasticity of euryhaline teleosts to saltwater is correlated with a significant increase in Na^+/K^+-ATPase activity in the ionocytes of the gills (Epstein et al. 1967). In addition, the gills and epithelia of teleost fish express cystic fibrosis transmembrane conductance regulators (CFTR), a low conductance anion channel that plays a significant role in the excretion of saltwater in the gills (Marshall and Singer 2002). Thus, significant reconstruction of the gill epithelium must take place

in order for fish to make the transition from freshwater to saltwater and vice versa (Evans et al. 2005, *see review by* Dymowska et al. 2012).

Previous work indicates that euryhaline fishes do, in fact, exhibit plastic responses in ionocyte subunits when faced with salinity changes in their ambient environment, and this is aided by the endocrine system (Hiroi and McCormick 2012; Mackie et al. 2007; Pelis and McCormick 2001). Cortisol, for example, was originally known to promote survival and osmoregulation when fish acclimate to saltwater (Mommsen et al. 1999). However, Laurent and Perry's (1990) study on ion uptake in freshwater trout (*Salmo gairdneri*) has shown that cortisol is also involved in increased Na^+ and Cl^- uptake for fishes in freshwater environments. Thus, cortisol is important for both freshwater and marine fishes, but its role in osmoregulation and adaptation differs between the two types. For example, previous research has shown that in freshwater, prolactin and cortisol interact to promote the differentiation of stem cells into freshwater ionocytes, whereas in saltwater cortisol interacts with GH/IGF-1 axis to differentiate stem cells into saltwater ionocytes (Figure 1.2) (McCormick 2011; Schultz and McCormick 2012; McCormick and Bradshaw 2006). Changes in salinity, leading to changes in internal osmolality triggers the release of cortisol from the Hypothalamus-Pituitary-Interrenal (HPI) axis into the blood plasma to allow for acclimation to occur (McCormick 2011; Tort 2011).

In most vertebrates, cortisol (or corticosterone) is the primary glucocorticoid and binds specifically to the glucocorticoid receptor (GR) whereas aldosterone, the primary mineralocorticoid, binds specifically to the mineralocorticoid receptor (MR). Since fish either lack aldosterone completely or contain trace amounts of the mineralocorticoid, there is support for the concept that cortisol plays a dual role as both a glucocorticoid (to influence both metabolism and growth) and mineralocorticoid (for water and ionic regulation) (McCormick and Bradshaw 2006).

Therefore, it is not surprising that acute and chronic stress have been shown to affect (increase or decrease) the metabolic rate of fishes (*see review by* Wendelaar Bonga 1997). When a fish is subjected to a salinity stress, osmoregulatory mechanisms must be employed and energy is expended (Ern et al. 2014), wherein metabolism, and thus growth and activity are negatively affected (Varsamos et al. 2005).

Increases in energy expenditure allocated to osmoregulation can thus create a potential trade-off between acclimation ability to salinity changes and other traits associated with fitness (i.e., growth, activity, swimming performance, etc.). In fact, such trade-offs between tolerance to an abiotic stressor and fitness-associated traits have been documented before (Swanson 1998; Johnson et al. 1998). Therefore, salinity acclimation may come at the cost of performance for other traits, posing negative impacts for the survival and persistence of a species or population in a given environment.

Despite the potential for such trade-offs to shape distribution patterns across salinity gradients, relatively few studies have explored how osmoregulation and energy expenditure shape salinity preferences. For example, flounder, *Paralichthys orbignvanus*, typically inhabit coastal shores and estuaries. A study conducted by Sampaio and Bianchini (2002) showed that although these fish can survive and grow in freshwater, they typically grow at a slower rate than those individuals that were reared in salt and brackish waters. They speculate that this could potentially be due to the increase in energy expenditure that is necessary for osmo- and ionoregulation in freshwater versus water with higher levels of salinity. This reduction in growth rate could potentially be the reason behind their absence of freshwater environments in the wild, as a decrease in growth is typically associated with having a competitive disadvantage (Mittelbach 1988). This is a primary example

of how local adaptation to a particular salinity could impose constraints on an organism, preventing the organism from expanding its range.

Overarching Question and Approach

In this dissertation, I sought to understand range limits for two closely related euryhaline species that differ in their distributional patterns along a salinity gradient. This goal was accomplished by investigating potential trade-offs between salinity tolerance and fitness-related traits. Hormonal analyses were also conducted to provide insight to plasticity for a given salinity range along the gradient.

Study System

The distribution of euryhaline fish species along salinity gradients provides an ideal opportunity to investigate how trade-offs and plasticity shape distributional limits. On the island of Trinidad, the guppy (*Poecilia reticulata*) is restricted to freshwater (0 psu) habitats, while the swamp guppy (*Poecilia picta*) is found in brackish and saltwater (1+ psu) environments, but also overlaps guppies in freshwater sites (Figure 1.3). This pattern is repeated in rivers and streams throughout the island as water flows the inland to the coasts (Torres-Dowdall et al. 2013). Guppies are considered euryhaline and are known to occupy fresh and brackish water habitats throughout their geographic range (Rosen and Bailey 1963), suggesting trade-offs between salinity tolerance and other fitness-related traits (e.g., food acquisition, competition, and escape behavior) might jointly explain why guppies are restricted to freshwater in Trinidad (Torres-Dowdall et al. 2013).

The Trinidadian guppy is an extensively studied model system (Carneiro et al. 2007; Magurran 2005; Reznick and Endler 1982; Endler 1995) and much is known about their distribution patterns

(Magurran 2005; Torres-Dowdall et al. 2013) as well as how predatory regime can influence the evolution of various traits in guppies associated with coloration (Magurran 2005), behavior (Ioannou et al. 2017), morphology (Hendry et al. 2006), and life history characteristics (Reznick and Endler 1982). However, locomotor performance is relatively understudied (*but see* Ghalambor et al. 2004). Although they do not naturally occur in brackish habitats in Trinidad, previous studies have investigated the ability of guppies to survive both abrupt and gradual changes in salinity in a lab setting (Chervinski 1984; Shikano and Fujio 1997; Shikano et al. 2001). For example, Shikano and Fujio (1997) conducted saltwater tolerance studies on guppies and found that gradual acclimation is required for fish to tolerate substantial overall changes in salinity levels. Survival rates began to decline significantly when fish were taken from 0 psu environments and exposed to salinity levels above 20 psu. Therefore, although they are a euryhaline species, they do not seem to acclimate well to rapid changes in salinity or very high levels of salinity.

The swamp guppy, conversely, is relatively understudied. Very little is known about this species in terms of their ecology, physiology, and behavior. However, we do know that fresh and brackish swamp guppies are morphologically similar in terms of their relative size and coloration (Moyer, Marshall, and Ghalambor, *unpublished*), but whether or not these ecotypes are locally adapted to their particular environment is unknown. Therefore, although this species is found in fresh, brackish, and saltwater habitats, how they use salinity acclimation and behavioral avoidance of salinity changes along that gradient is unknown.

Previous work from the lab has indicated that interspecific interactions between both species affects growth rate and indicates that, when resources are limited, the guppy could potentially be excluding the swamp guppy from extending its range further upstream in freshwater habitats. However, resources do not seem to be limited for both species in their environment. In fact, field

observations suggest that we might see a partitioning of resources where the two species overlap. Therefore, the answer to their repeated patterns of dispersal might lie in their ability to physiologically tolerate changes in salinity. In a lab setting, we know that both species can acclimate to salinity levels of at least 30 psu, however this is in a controlled setting nonrepresentative of natural conditions. In the lab, they are void of predators, their food resources are abundant, intraspecific competition is low, and interspecific competition is non-existent. This is not the case in their naturally occurring habitats.

To understand patterns of dispersal for both species, I employed an integrative approach to investigate the potential trade-offs that might be occur as a result from acute salinity exposure that these fish might experience on a daily basis due to tidal fluctuations. Specifically, I sought to understand the mechanisms that allow swamp guppies to occupy the full salinity gradient, but restrict guppies to freshwater. I examined how salinity influences swim performance (i.e., U_{CRIT} and burst swimming) and the potential trade-off between performance and the ability to maintain internal osmolality (Chapters 2 and 3). I also quantified differences in free cortisol in swamp guppies to make inferences about plasticity along a salinity gradient while confirming that daily salinity stress results in elevated cortisol levels (Chapter 4).

TABLES & FIGURES

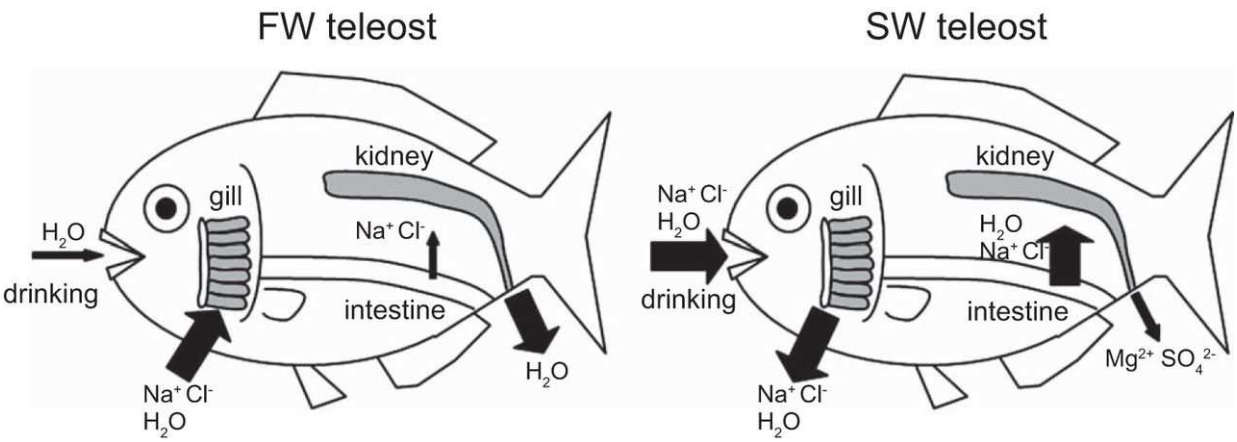

Figure 1.1. Diagram depicting differences in osmoregulatory challenges in freshwater (FW) versus saltwater (SW) teleosts (from Marshall and Grosell 2006).

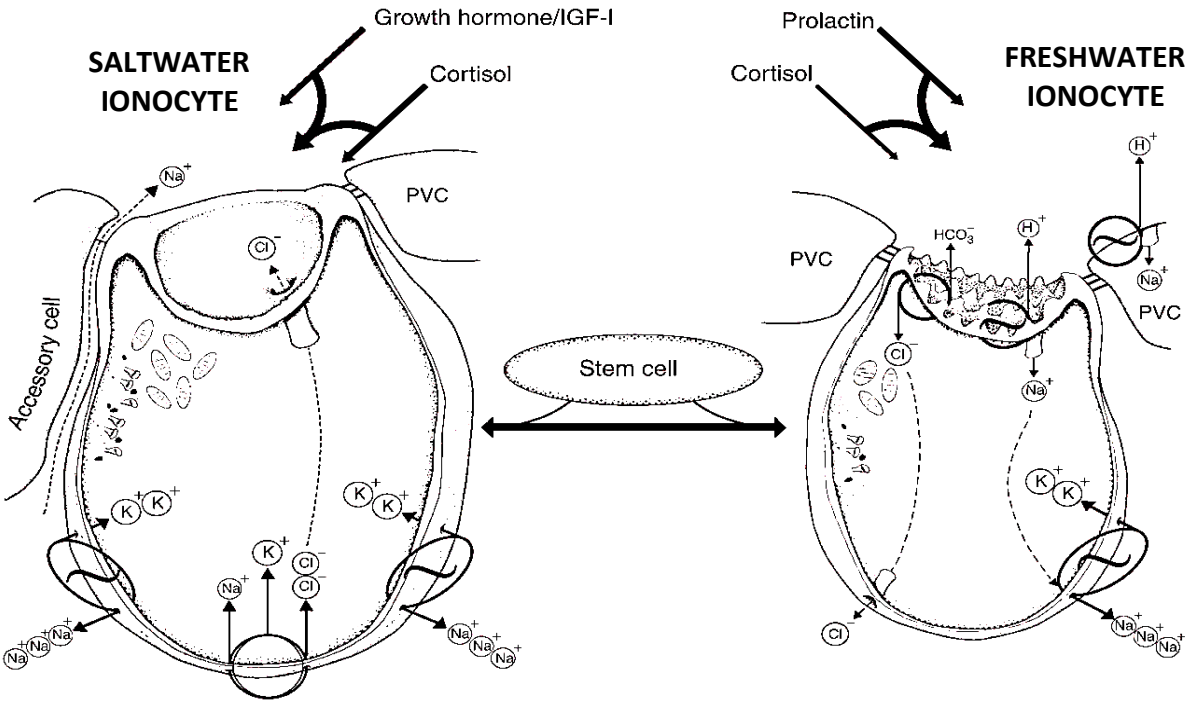

Figure 1.2. Diagram depicting the dual role of cortisol in promoting acclimation to both SW (right) and FW (left) (from McCormick 2011).

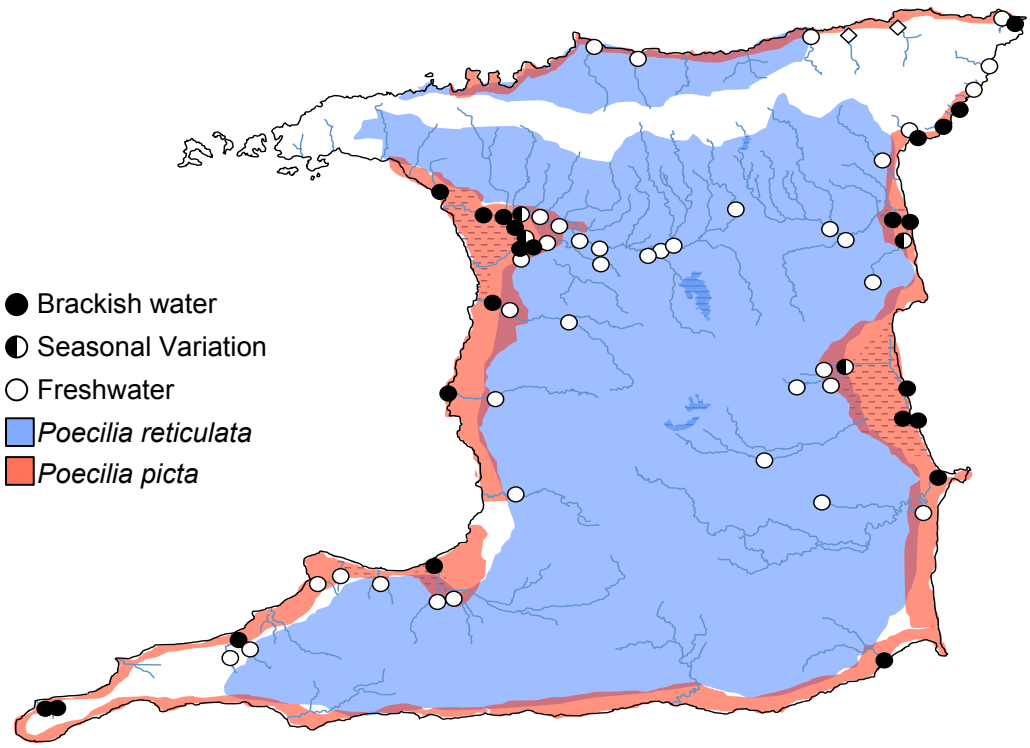

Figure 1.3. Diagram depicting the geographic distribution of *P. picta* and *P. reticulata* along a salinity gradient on the island of Trinidad (from Julian Torres-Dowdall).

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CHAPTER 2: SALINITY PERFORMANCE CURVES AND OSMOREGULATORY CAPACITY FOR SUSTAINED SWIMMING PERFORMANCE (U_{CRIT}) IN A MALE EURYHALINE FISH

INTRODUCTION

A fundamental goal of eco-physiology is to better understand how integrated traits (i.e., locomotion, growth, feeding efficiency, etc.) contribute to whole organismal performance and in turn shape ecological patterns in nature (Irschick and Garland 2001; Irschick et al. 2008; Lailvaux and Husak 2014; Lailvaux and Husak 2017; Orr and Garland 2017; Arnold 1983). Because performance is correlated with Darwinian fitness (Arnold 1983; Le Galliard et al. 2004; Husak et al. 2006; Walker et al. 2005) how performance varies across abiotic gradients provides insight into the physiological limits of tolerance and the range of conditions performance is optimized (Huey and Stevenson 1979; Sinclair et al. 2016, Huey & Kingsolver 1989, Spicer and Gaston 1999, Pörtner et al. 2006, Angilletta 2009). Temperature, for example, is known to affect biological processes from the cellular to the organismal level in ectotherms (Somero 2012; Schulte 2015). As a result, whole organism performance varies as function of temperature due to the way biochemical reactions, cellular metabolism, nervous system function, and protein stability respond to increasing temperature (Schulte 2015; Schulte et al. 2011; Haesemeyer 2020). Thus, thermal performance curves (TPCs), where changes in performance are elucidated through subjecting individuals to increasing or decreasing temperatures, can be useful to understand the range of "optimal" temperatures, which in turn can provide insight into ecological patterns in nature (i.e., distributional patterns, thermal tolerance, and habitat preference) for a given individual or population (Schulte et al. 2011).

Salinity, like temperature, can influence biological processes from the cellular to whole organism level, and most aquatic species employ active osmoregulatory mechanisms to maintain

a constant internal osmolality (Evans et al. 2005; McCormick 2001). For osmoregulating fishes, maintaining osmotic and ionic homeostasis is essential for optimal function of cellular and physiological processes (Hochachka and Somero 2002; Evans et al. 2005; Hwang and Lee 2007). Teleost fishes maintain an internal osmolality ~300 mOsmol/kg, nearly a third the salinity of seawater (Kültz 2015). Therefore, freshwater (<0.5 psu) fishes face the challenge of absorbing Na^+ and Cl^- ions through their gill epithelium from their relatively dilute surroundings while minimizing ionic loss (Kumai and Perry 2012), whereas saltwater (~33-40 psu) fishes increase water intake as a means to replace water lost to their external environment while expelling large concentrations of salt ions (Wurts 1998). Efficient osmoregulation is critical, as significant deviations in blood plasma osmolality cause changes in cellular volume and thus function, leading to mortality (Lutz 1972). Similar to behavioral thermoregulation (Neill 1979), fish can employ behavioral strategies to avoid salinity fluctuations as a means to reduce energy required for osmoregulation (Edeline et al. 2005; Dowd et al. 2010).

While about 95% of teleost species are stenohaline and live exclusively in either fresh or saltwater, the remaining 5% are euryhaline species, capable of tolerating, and thus inhabiting, a wide range of salinities (Evans 1984). Truly euryhaline species must actively regulate internal plasma ions when faced with changes in salinity (McCormick 2011; Evans and Claiborne 2008), requiring a significant reconstruction of the gill epithelium including gill co-transporters, enzymes, and ionocytes. The ability to employ these mechanisms allows euryhaline fish to successfully transition from freshwater to saltwater and vice versa (McCormick et al. 2003; Evans et al. 2005; Marshall and Grosell 2005; *see review by* Dymowska *et al.* 2012). Since crossing along a salinity gradient typically presents an osmoregulatory challenge (Kültz et al. 1992), the freshwater-saltwater interface can act as a barrier delineating the range limits of some euryhaline species.

In the face of a salinity challenge, increased energy allocated to osmoregulation will decrease the amount of energy that can be allocated to other aspects of aerobic performance, such as growth, foraging, mating, and other energetically demanding activities (Boeuf and Payan 2001; Calow and Forbes 1998; Sokolova et al. 2012). The energetic demands of osmoregulation in the face of a salinity challenge can thus lead to a trade-off between acclimation ability and other traits associated with fitness (i.e., foraging, mate acquisition, and swimming performance). Thus, although physiologically capable of tolerating salinity changes, negative impacts that result from increased energy expenditure necessary for osmoregulation can lead to some physiologically euryhaline fish being ecologically stenohaline. Most research investigating the effect of salinity on physiological processes (i.e., metabolism, osmoregulatory costs, or growth potential) in euryhaline fishes has occurred in isolation without considering how increased energy expenditure can impact behavioral traits such as changes in sustained swimming performance (U_{CRIT}) (*but see* Haney and Nordlie 1997; Swanson 1998; Plaut 2000). However, previous work has emphasized the trade-off between osmoregulation and respiration, termed the osmo-respiratory compromise, in which optimal conditions of the gill necessary for increased oxygen uptake (i.e., increased gill profusion) comes at the expense of ion regulation, and vice versa (Sardella and Brauner 2007). Thus, a salinity challenge that leads to decreased metabolic efficiency may cause a decrease in swimming performance, negatively affecting fish health and survival.

A powerful approach for testing potential energy trade-offs is to measure salinity performance curves (SPCs) to understand how performance changes in the face of an osmoregulatory challenge (Figure 2.1). Given that there can be substantial inter- and intraspecific variation in factors that determine how quickly fish acclimate, including the rate of change in gene expression and time course necessary for proper gill reconstruction, the shape of SPCs can vary significantly (see

Figure 2.1). For example, efficient osmoregulatory capacity and rapid acclimation might lead to stable performance across a wide range of salinities, while reduced osmoregulatory capacity might lead to decreased performance outside of the natural salinity environment and suggest a potential trade-off between maintaining performance in the face of increasing osmoregulatory demands. Alternatively, for teleosts experiencing an isosmotic environment, where their internal osmolality matches their external environment (~12 psu), it might be expected that reduced energy expenditure for osmoregulation will lead to optimal levels of performance (Figure 2.1). However, relatively few studies have examined the shape of SPCs or attempted to use them to explain habitat use or ecological patterns in natural populations.

Euryhaline fishes provide a unique opportunity to investigate the effects of salinity acclimation on whole-organism performance. Previous work on Trinidadian guppies, *Poecilia reticulata*, confirms they are euryhaline and can survive both abrupt and gradual changes in salinity in a lab setting (Chervinski 1984; Shikano and Fujio 1997; Shikano et al. 2001). However, on the island of Trinidad, the guppy exhibits behavioral avoidance of salinity in natural streams (Torres-Dowdall et al. 2013; Mauro et al. *in review*). Guppies are known to occupy fresh and brackish water habitats throughout their geographic range (Rosen and Bailey 1963), suggesting that despite exhibiting salinity tolerance (i.e., osmoregulatory capacity) they may experience a decline in other fitness-related traits (e.g., food acquisition, competition, and swimming performance) which might explain why guppies are restricted to freshwater in Trinidad (Torres-Dowdall et al. 2013).

Here, we seek to determine the effect of salinity changes on fitness related parameters by generating a salinity performance curve for critical swimming velocity (U_{CRIT}) and osmoregulatory capacity. We hypothesized that decreased swimming performance, caused by an inability to rapidly acclimate to increased levels of salinity, may be a factor in preventing guppies from

persisting beyond freshwater environments in Trinidad. We tested this hypothesis by collecting guppies from a freshwater site, acclimating them to increasing levels of salinity, and measuring their swimming performance and plasma osmolality. We predicted that if guppies exhibit a decline in swimming performance in the face of increasing salinity, they would also have a corresponding inability to maintain a constant plasma osmolality. Alternatively, if swimming performance is maintained across salinities, we predicted the maintenance of a constant plasma osmolality concentration (~12 psu).

METHODS

Animal Collection and Husbandry

We collected wild adult male *P. reticulata* (n=80) in March 2020 from a freshwater (0 psu) portion of the Caroni River (Lat: 10.6207; Long: -61.4567) on the island of Trinidad. Due to the effect of pregnancy on sustained swimming performance (Ghalambor et al. 2004), we only focused on male fish during the U_{CRIT} experiments. Adult males were transported to the field station in Manuel Congo, Trinidad where they were placed into one of two 20-gallon freshwater (0 psu) aquaria so that numbers did not exceed 40 individuals per tank. Aquarium water was pre-treated with Kordon AmQuel® Plus, API® Stress Coat, and PraziPro™ to rid the water of unwanted/harmful chemicals and cleanse the fish of parasites. Fish were fed API® Tropical Greens flakes *ad lib* once daily. Individuals were kept in group tanks for at least 72 hours before beginning the experiment to carefully observe and weed out any fish that may have been sick or injured.

Salinity Acclimation

We tested the effect of an increase in salinity on sustained swimming performance. To do so, fish were acclimated incrementally from freshwater to their test (or trial) salinity. Acclimations and trials were performed sequentially from least to highest salinity (0 psu, 6 psu, 9 psu, 12 psu, 15 psu, and 18 psu) so that we tested one salinity per day. A total of 60 males were used in this experiment and each individual was only subjected to one specific test salinity. We tested 10 males at each salinity over the course of two days per salinity (5 individuals per day; 10 unique individuals per salinity). Fish remained in group tanks until 24 hours before their trials began. Each day, 5 individuals were removed and transferred to individual 1-gallon containers consisting of pre-treated water reflective of their test salinity. We staggered the time that fish were removed from the group tank and placed in an individual container to ensure they did not acclimate to the test salinity longer than approximately 28 hours before the swimming trial. Once in an individual container, fish were not fed to ensure a standardized post-absorptive state during the trail. Once all experimental fish were removed from the stock tank for a particular salinity, we increased the salinity of the stock tanks to allow remaining fish to achieve a gradual acclimation up to their specific test salinity (see Table 2.S1). Thus, fish in the stock tanks were experiencing an increase in salinity every two days. For example, fish being tested at 9 psu were acclimated up to 6 psu in their group tanks before being subjected to an acclimation salinity of 9 psu in their individual 1-gallon container 24 hours prior to their trial. Once individuals were transferred to the experimental apparatus, their 1-gallon holding containers were emptied, sanitized with 90% EtOH, and rinsed for reuse in the next batch of experimental fish.

Swimming Performance

Critical swimming speed (or U_{CRIT}) which captures the maximum sustained swimming velocity was measured using a Loligo swim tunnel system. Fish were transferred from their individual container to a 170 mL swim tunnel chamber (Loligo Systems, Denmark) with an open flow to prevent depletion of oxygen in the tunnel (see Figure 2.2). The experimental apparatus was covered in black plastic to decrease stress from visual movements and prevent distraction during the acclimation and test period. We allowed the fish to acclimate to the apparatus with the propeller set to a low velocity (1.5 cm/s) for a period of 60 minutes prior to the beginning of the trial. All trials were carried out at 30˚C.

Following the 1-hour acclimation period, fish were subjected to a U_{CRIT} protocol modified from Oufiero et al. 2011. The propeller speed was increased to 10 cm/s to start the trial, as velocities below 10 cm/s did not encourage swimming. Fish were monitored to ensure that they were swimming rather than resting against the bottom or back of the swim tunnel chamber. Velocity within the swim tunnel was increased by 1.5 cm/s every 5 minutes until the fish could no longer maintain its position in the apparatus. Exhaustion was denoted once a fish could no longer pull itself from the barrier at the back of the swim tunnel after a period of 3 seconds. The highest completed velocity (highest velocity where swim duration lasted the entire 5 min period), swim duration in the final achieved velocity, and total swim duration were recorded.

U_{CRIT} was measured using the following equation from Brett (1964): $U_{CRIT} = U_f + [U_i(T_f T_i^{-1})]$, where U_f represents the highest completed velocity (cm/s), U_i is the increment at which water velocity was increased in a step-wise manner (1.5 cm/s), T_f is the swimming duration during the final achieved velocity (cm/s), and T_i is the duration the fish swam at each velocity (300 s).

Upon completion of their trial, fish were immediately dried using a Kimwipe® and weighed to obtain mass (g). After weighing, fish were placed on white background with a scale and photographed to obtain length measurements using ImageJ.

Quantifying Osmolality

In the lab at Colorado State University, we used second generation (F2) lab born fish of both sexes to quantify how the acclimation scheme outlined above affects internal osmolality for each species. These fish were originally born and reared in freshwater (0 psu), where they remained throughout their entire duration in the lab. The total number of fish available were split equally into 6 groups (~6-7 individuals per group) and then transferred into separate 2.5-gallon aquaria. After a 2-day acclimation to the new tank, fish from one freshwater aquarium were euthanized to obtain blood plasma following the procedure outlined below. All individuals in other aquaria were subjected to the same salinity acclimation procedure described above for wild-caught fish, with increases in salinity occurring every 24 hours and euthanasia of one group at each salinity.

Blood plasma was extracted following methods described in Havird et al. (2014) to increase plasma extraction volume compared to other conventional blood extraction methods (e.g., syringe, caudal peduncle clipping, etc.). Specifically, fish were transferred to a dry, non-porous surface and the exterior of each individual was gently dried with a Kimwipe®. Once dried, each fish was decapitated using a straight-edge blade, and their brains were pithed to ensure rapid euthanasia. Following decapitation, fish were immediately transferred (exposed side down with caudal fin facing upward) into a 0.22um centrifuge filter tube and spun down for 12 min at 13,400 rpm. Limiting the time between decapitation and the centrifuge process reduced blood coagulation. Using a filter tube allowed tissue to separate from fluids to ensure that fluids were not

contaminated. The filters and fish remains were removed from the tube, and tubes containing internal fluids were then transferred to -20˚C prior to osmolality quantification.

After salinity acclimation was completed for all groups, samples were defrosted and centrifuged for 5 minutes at 13,400 rpm to separate plasma from red blood cells. Two microliters of plasma from each sample were transferred via a 2uL - 10uL pipette to a Wescor Vapor Pressure Osmometer to quantify osmolality (mmol/kg).

Statistical Analysis

U_{CRIT}. Our goal was to estimate U_{CRIT} for each test salinity, so that we could generate a SPC across different salinities. However, our experimental design tested different individuals at each salinity following sequential acclimations to higher levels of salinity, thus we were unable to account for individual level effects on U_{CRIT}. Measurements of body mass (g) and body length (cm) were used to calculate Fulton's body condition (g/cm^3) (Neumann et al. 2012) for each individual and mean values were recorded for each salinity (Table 2.S2). To estimate the potential effect of size on U_{CRIT}, we used body mass (g) as a covariate, as it was significantly correlated with length ($r = 0.74$, $p < 0.0001$). We investigated the heterogeneity of slopes using an analysis of covariance (ANCOVA) that first tested only body mass and the interaction between body mass and test salinity. We found body mass was a significant covariate ($F_{1,53}=4.37$, $p=0.041$), but the interaction was not significant ($F_{5,53}=1.58$, $p=0.180$). Therefore, we re-ran the model without the interaction. We conducted two post-hoc pairwise comparisons 1) between 0 psu and 6 psu to test the a priori hypothesis that an initial change in salinity should result in decreased performance, and 2) between 12 psu and each of the other salinities individually to test the a priori hypothesis that performance is maximized at the isosmotic point. We then plotted the estimated marginal mean values of U_{CRIT}

(±95% confidence intervals) for each salinity to estimate the shape of the SPC. All statistical analyses were performed in R with a critical value for significance of 0.05.

Osmolality. We used a linear model to determine the relationship between test salinity and osmolality. Although male and female guppies were represented in the osmolality experiment, a two-way ANOVA revealed sex was not significant in the model ($F_{1,30} = 0.55$, $p = 0.463$), and was thus not included in the statistical analyses. Although not included in our final model, conclusions about the effects of salinity were similar regardless of including sex in the model. The `emmeans` package in R was used to generate estimated marginal means for the effects salinity on osmolality, and Tukey adjusted pairwise comparisons between the six salinity treatments were conducted using the `pairs()` function in R.

RESULTS

U_{CRIT}

The influence of body mass on U_{CRIT} was significant ($F_{1,53} = 4.28$, $p = 0.043$), but the main effect of salinity was not ($F_{5,53}=1.74$, $p=0.141$). While there was a trend for performance to decrease between 0 psu and 6 psu, this trend was not significant ($p = 0.447$). However, we did find U_{CRIT} was highest at 12 psu and significantly different from 6 psu ($p = 0.010$), 9 psu ($p = 0.048$), 15 psu ($p = 0.005$), and 18 psu ($p = 0.026$) using one-tailed tests; see also Figure 2.3 and Table 2.S3).

Osmolality

Osmolality was measured for 6-7 individuals for each of the experimental salinities (Figure 2.4; Table 2.S4). Tukey adjusted pairwise comparisons revealed that there were no significant

differences between any of the salinity treatments (all $p > 0.1$). However, there was a non-significant increase from 0 psu to 6 psu to 9 psu, and osmolality remained high for fish acclimated to 12, 15, and 18 psu (Figure 2.4).

DISCUSSION

Performance curves provide a relatively simple but powerful way of testing how aspects of the external environment influence the physiology of an organism and in turn whole organism performance (Huey and Stevenson 1979; Sinclair et al. 2016, Huey and Kingsolver 1989). Here, we investigated the shape of the SPC to test the effects of increasing salinity on swimming performance and osmoregulatory capacity in a euryhaline fish that is ecologically stenohaline (i.e., confined to freshwater) on the island of Trinidad. Overall, we found *P. reticulata* was able to maintain sustained swimming performance (U_{CRIT}) across a broad range of salinities, although there was a clear peak at the isosmotic salinity of 12 psu (Figure 2.3). Collectively, these results suggest *P. reticulata* is able to meet the competing demands of osmoregulation and sustained swimming, and although confined to freshwater, achieves maximum performance at the isosmotic salinity. In a separate experiment, we subjected lab-reared fish to the same salinity challenge as individuals in the swimming performance trials, and found no significant change in plasma osmolality, suggesting a well-developed osmoregulatory capacity (Figure 2.4).

Our estimates of how salinity increases impact U_{CRIT} support the general consensus that *P. reticulata* is euryhaline and is able to maintain sustained swimming performance across a range of salinities (see Figure 2.1). Similar studies of other euryhaline fish have found similar results. For example, the transfer from saltwater to freshwater in European seabass (*Dicentrarchus labrax*) (Chatelier et al. 2005) and from freshwater to saltwater in tiger puffers (*Takifugu rubripes*) (Yu et

al. 2018) resulted in no significant change in U_{CRIT}. However, salinity challenges have been shown to elicit significant declines in swimming performance (U_{CRIT}) in both juvenile coho salmon (*Oncorhynchus kisutch*) (Brauner et al. 1994) and Adriatic sturgeon (*Acipenser naccarii*) (McKenzie et al. 2001) upon being transferred to saltwater. Although, in both coho salmon and Adriatic sturgeon, a reduction in swimming performance was a correlated with significant increases in both osmolality and plasma ion concentrations (McKenzie et al. 2001; Brauner et al. 1992), suggesting that while they are anadromous, they may not have the osmoregulatory capacity of fully euryhaline fish species. In fact, significant increases in plasma osmolality have been linked to a reduction in whole-animal performance, including swimming performance for other fish species (Penny and Kieffer 2019).

The ability to maintain a consistent osmolality is critical for biological processes, physiological homeostasis, and survival (Kültz 2015), and maintaining a constant osmolality across a range of salinities provides strong evidence for robust osmoregulatory mechanisms. While there was a non-significant trend for an increase in osmolality when *P. reticulata* was transferred from freshwater to brackish water, consistent with the swimming performance, there was no indication increasing salinity compromised ion homeostasis. Previous work in fundulid fishes has revealed extensive variation among closely related euryhaline species in their ability to recover during an osmotic challenge (Whitehead et al. 2013, Yetsko and Sancho 2015). For example, *Fundulus heteroclitus* and *Fundulus majalis* both exhibited extensive gill modifications to offset an osmotic challenge in a transition from brackish to freshwater; however, only *F. heteroclitus* was capable of making a full transition to the freshwater gill phenotype and as a result was able to maintain its internal osmolality (Whitehead et al. 2013). In addition, Kidder and colleagues (2006) have shown that following transfer to higher salinities, *F. heteroclitus* initially exhibits a drop in activity and

metabolic rate, but then is able to maintains a consistent resting metabolic rate without increased oxygen consumption. Such results suggest that either energy for osmoregulation is made available at the expense of another physiological or biochemical process, or *F. heteroclitus* rapidly acclimates and does not require additional energy for osmoregulation. Without metabolic data from fish in this experiment, we unfortunately cannot make claims about the energy expenditure or costs of osmoregulation for *P. reticulata* subjected to a salinity increase. However, previous work has shown that different strains of *P. reticulata* vary in their ability to maintain osmolality and survive (Shikano and Fujio 1997), suggesting osmoregulatory costs may vary between populations.

One limitation of this work was the inability to obtain plasma samples from the same fish that were used in the U_{CRIT} trials. Given that male fish from the wild have substantially less mass than lab-reared fish, blood plasma volumes obtained from fish used in U_{CRIT} trials were not sufficient (i.e., less than 2 uL) to run on the Wescor Vapor Pressure Osmometer. The ability to correlate plasma osmolality with swim performance for a particular individual might have elucidated a potential trade-off since blood samples would have been collected from exhausted individuals rather than those from this experiment that were essentially measured at rest.

It is also important to note that our conclusions were based solely on U_{CRIT} measurements in male fish, and thus we cannot say for certain that females would show a similar pattern. Given their larger body size and less streamlined shape, we might expect females to exhibit reduced swimming performance at increased speeds and salinities. Future research could explore whether swim performance of females is compromised with changes in salinity, contributing to *P. reticulata*'s freshwater distribution in Trinidad. In addition, while measuring U_{CRIT} provides insights into aerobic swim performance, which contributes to the ability forage and seek mates

(Plaut 2001), it represents only one metric of swimming performance. For example, a constant acceleration test speed (U_{CAT}), which forces fish to swim against a constantly increasing current utilizing anaerobic metabolism, can be used to understand their ability to escape chasing predators (Nelson et al. 2002; Oufiero and Garland 2009; Marras et al. 2010). Alternatively, burst swim performance could also be measured to provide insight into the ability of fish also to escape predation in their natural environment. Since *P. reticulata* are also common prey for sit-and-wait predators, burst swimming performance can also be measured to quantify aspects of an anaerobic startle response (Walker et al. 2005; Domenici 2010).

Conclusion. We find strong support for the euryhaline characterization of *P. reticulata*, as it can maintain sustained swimming performance and osmolality across a wide range of salinities, but with peak swimming performance observed at 12 psu, or the isosmotic point. Thus, we conclude that *P. reticulata's* avoidance of brackish water on the island of Trinidad is not due to a reduction in sustained swimming performance as measured by U_{CRIT}, but likely to be caused by other factors. Indeed, salinity appears to compromise other aspects of performance that make *P. reticulata* more vulnerable to predators (Chapter 3), competitors (Torres-Dowdall et al. 2013; Mauro et al. in review), and parasites (Robison 2018). Thus, the reasons *P. reticulata* avoids brackish water are unlikely to be directly related to their osmoregulatory abilities, but rather through a number of indirect effects with the biotic environment.

TABLES & FIGURES

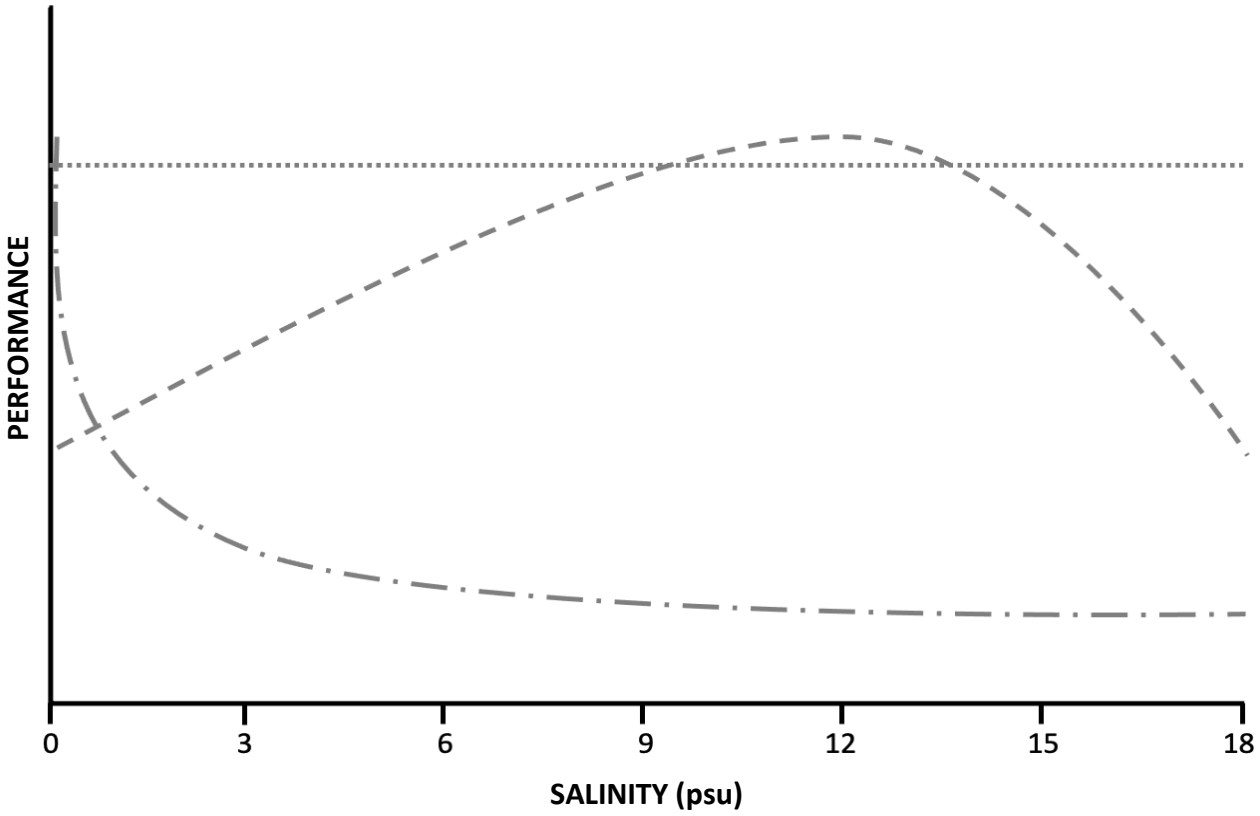

Figure 2.1. Alternative predictions of performance values along an increasing salinity gradient. (·····) represents optimal performance maintained along the gradient. (– – – –) represents optimality at the isosmotic point (~12 psu). (— · — ·) represents optimality in freshwater with a decrease in performance following a salinity increase.

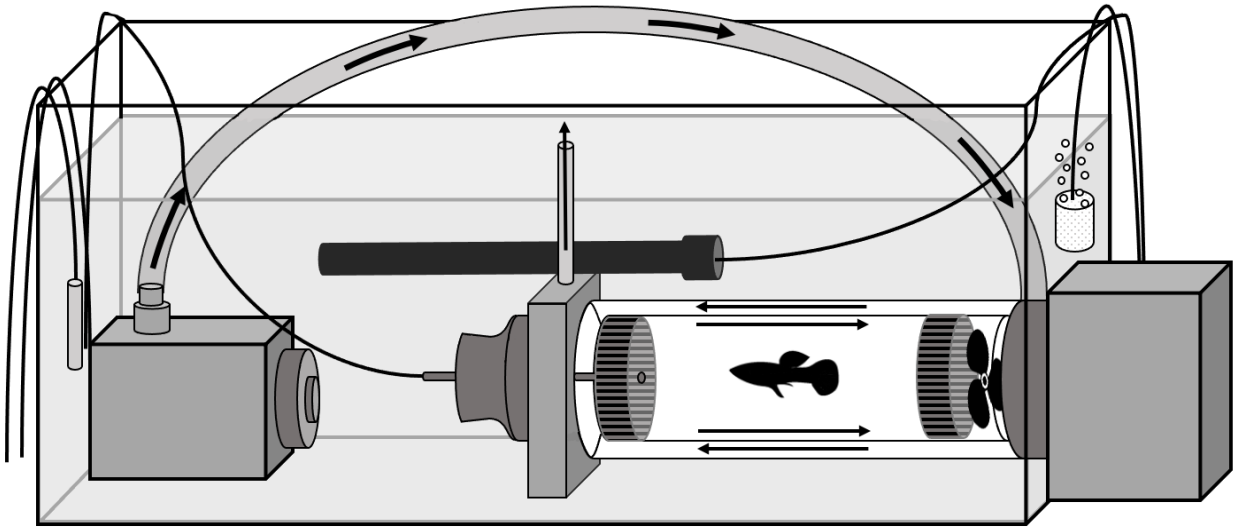

Figure 2.2. Experimental apparatus for U_{CRIT} measurements. The aquarium contained a swim tunnel, heater, temperature probe, water pump, and airstone. Arrows represent flow of water.

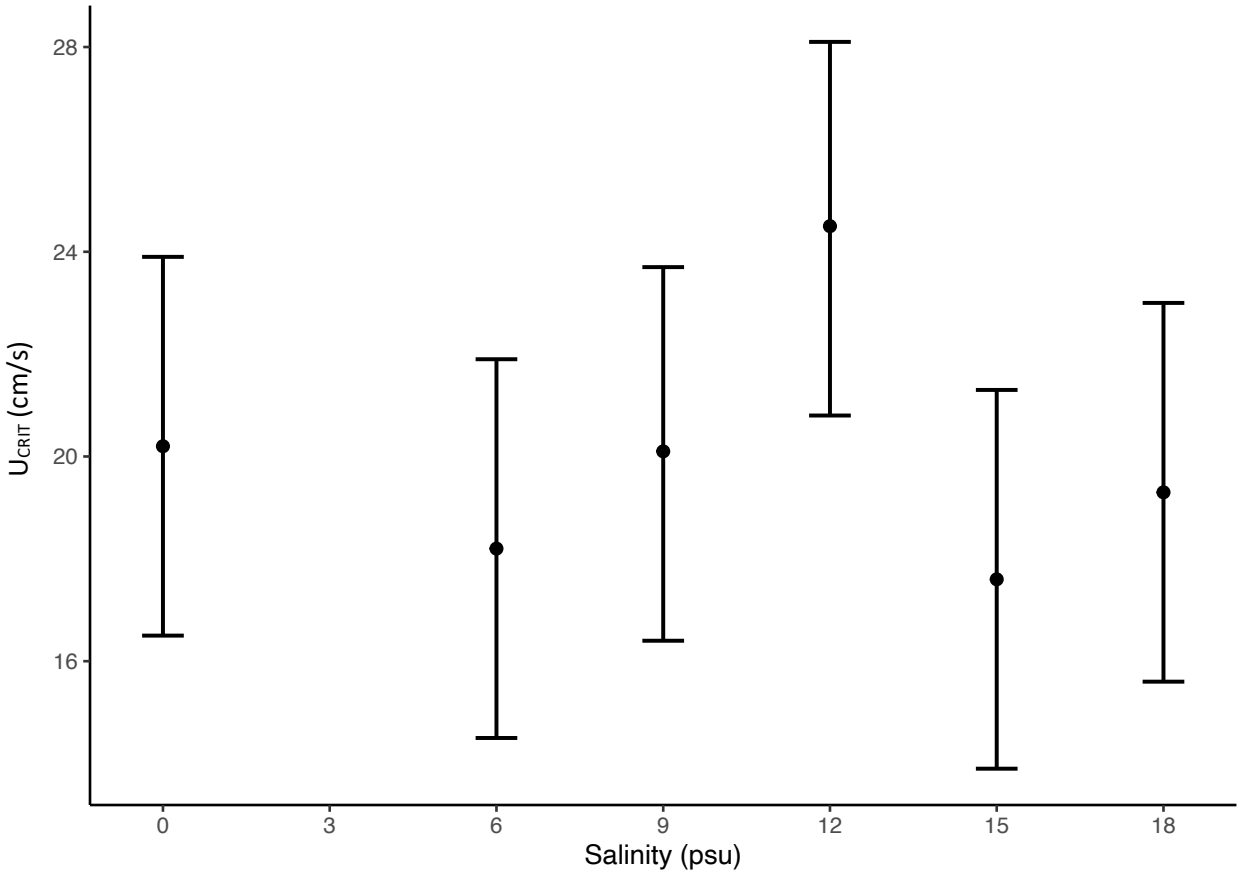

Figure 2.3. Depiction of relationship between U_{CRIT} (cm/s) values and salinity (psu). Estimated marginal means (EMMean ± 95% CI) are presented for each of the six test salinities. n=10 fish per salinity treatment.

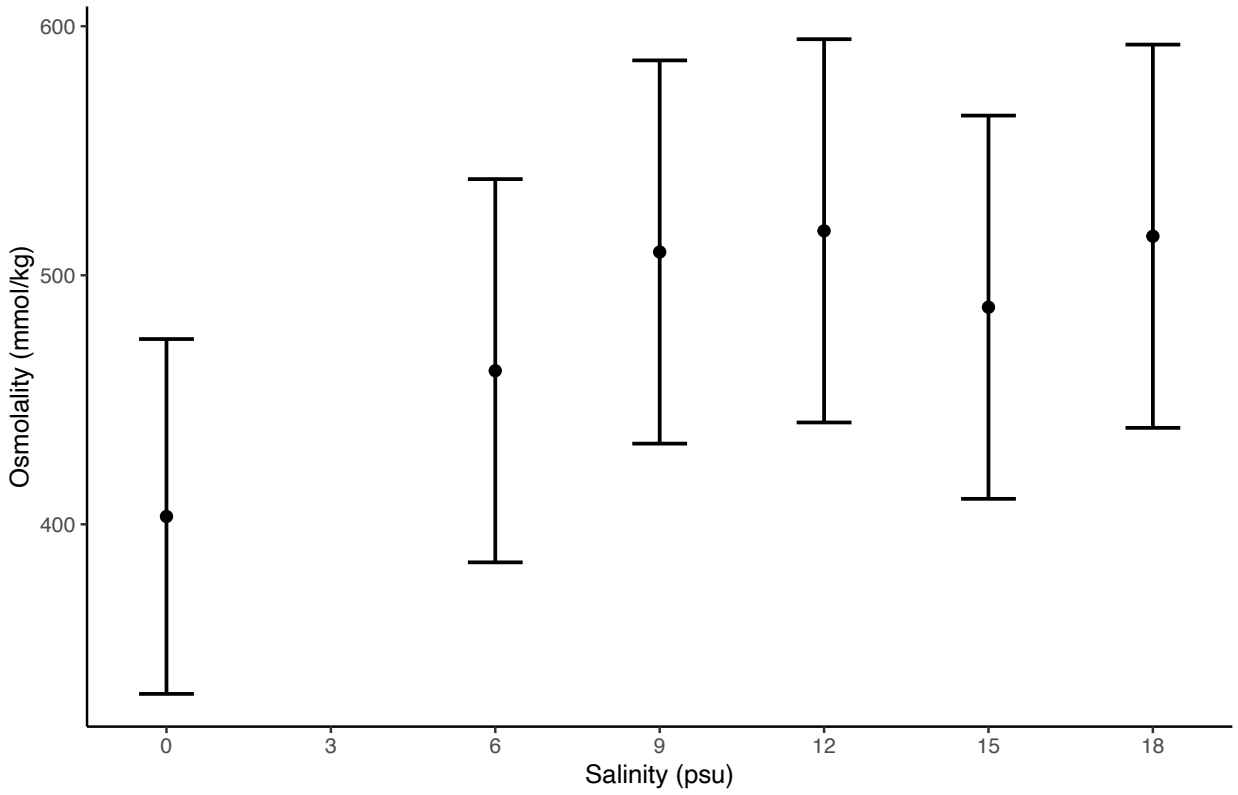

Figure 2.4. Depiction of relationship between blood plasma osmolality (EMMean ± 95% CI) for each of the six salinity treatments.

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CHAPTER 3: SALINITY PERFORMANCE CURVES FOR ESCAPE RESPONSE PROVIDE INSIGHT TO DISTRIBUTION PATTERNS IN TWO CLOSELY RELATED EURYHALINE FISHES

INTRODUCTION

A fundamental challenge in integrative biology is to understand the factors that shape variation in whole organismal performance and how this variation shapes the ecology and evolution of populations (Irschick and Garland 2001; Irschick et al. 2008; Lailvaux and Husak 2014; Lailvaux and Husak 2017; Orr and Garland 2017; Arnold 1983). Because variation in performance is related to fitness and is expected to be under strong selection (Arnold 1983; Le Galliard et al. 2004; Husak et al. 2006; Walker et al. 2005), considerable attention has been given to environmental sources of variation in organismal performance (Kingsolver and Gomulkiewicz 2003; Irshick 2003). In particular, the concept of a "performance curve" has been used to visualize and compare how performance changes as a function of an environmental variable. The power of such performance curves is that they estimate the range of conditions over which performance is maximized and minimized, providing key insights into habitat selection, the range of suitable environments, and vulnerability to environmental change (Huey 1991; Sinclair et al. 2016). For example, thermal performance curves commonly examine how some measure of performance (e.g., locomotion, growth, foraging) changes as a function of temperature (Huey and Stevenson 1979; Huey and Kingsolver 1989). However, performance curves can be measured for any other environmental factors that impact whole organism performance by influencing lower-level biological and physiological functions (Gunderson et al. 2016; Behrens et al. 2017; Sinclair et al. 2016). Although thermal performance curves are commonly studied in ectotherms to predict the effects of global climate change on species distributions and survival (Sinclair et al. 2016), variation in other environmental factors have also been examined. Salinity represents one such fundamental

enviornmental factor that impacts biological and physioloigal processes of many aquatic organisms (Evans et al. 2005; Marshall 2002; McCormick et al. 2013).

Many biological functions that influence locomotor performance have evolved to occur within a relatively narrow range of plasma osmolality (Moore and Gatten 1989) and aquatic organisms that experience both stable and fluctuating ambient salinity environments have numerous short- and long-term physiological mechanisms to maintain their internal osmolality (Kultz 2015). Such mechanisms are particularly important in euryhaline fish that often encounter a wide range of salinities in their environments (Lutz 1972; Kultz 2015). For example, many estuarine fishes experience fluctuating salinities due to tidal action and exhibit broad ranges of tolerance for salinity (Nordlie 2006). However, while a large body of research has uncovered the physiological mechanisms used to maintain ion balance in euryhaline fish (McCormick et al. 2013; Hiroi et al. 2008; McCormick 2001; Evans et al. 1999; Gaumet et al. 1995; Wood and Marshall 1994; Sampaio and Bianchini 2002), less is known about how variation in salinity impacts whole organism locomotor performance.

Burst swimming performance is essential for survival of fish species coexisting with predators (Walker et al. 2005; Domenici 2010); thus, research into escape responses (i.e., burst speed) in prey species has received considerable attention from fields ranging from neurophysiology (Eaton et al. 2001) to biomechanics (Domenici and Blake 1997; Wakeling 2006) and behavioral ecology (Godin 1997). In teleost fishes, a startle elicits a stereotypical behavioral response known as a C-start or fast-start, in which a unilateral muscle contraction bends the body into a C-shape followed by a contralateral muscle contraction (Domenici 2010). The C-start response is controlled by a pair of reticulospinal neurons known as Mauthner cells that are stimulated by sensory stimuli (i.e., visual, auditory, or olfactory cues) (Eaton et al. 2001; Domenici 2010) and is typically followed

by the high-energy propulsive stroke, accelerating the fish in the opposite direction of the stimulus. Thus, non-locomotor (e.g., detection and reaction time) and locomotor (e.g., maximum acceleration) performances jointly determine a fish's ability to successfully escape predation (Walker et al. 2005; Domenici 2010). Yet, few studies have investigated how variation in the abiotic environment alters these components of the escape response despite the obvious implications for individual fitness.

Escape performance can be influenced by a variety of abiotic factors, including temperature and salinity, that alter either the physical environment and/or lower-level biochemical, cellular, and physiological processes within the organism. Temperature can not only impact physical properties of the water, including viscosity and density which can resist movement, but also muscle contractile activity to jointly affect the escape response of a fish (Johnson et al. 1998). Less is known about how salinity influences burst swimming performance, but various lines of evidence suggest several potential factors. First, water viscosity increases with salinity, which could, in theory, negatively impact performance of small fish (Videler 1993; Johnson et al. 1998); however there is little empirical evidence in support of this (e.g., Christensen et al. 2018). Second, Grove and colleagues (2005) have shown that increases in osmolality negatively affect protein function, particularly the electrostatic interactions between actin and myosin filaments in skeletal muscles that are critical to the muscle contractions that generate the escape response. More generally, even minor shifts in osmotic balance can reduce performance and even be lethal (Lutz 1972; Evans et al. 2005; Christensen et al. 2019). Thus, changes in the physical properties of water combined with the challenge of maintaining homeostatic ion balance could alter escape performance, and negative effects of salinity on predatory response can lead to decreased survival, preventing fish from surviving and persisting in novel salinity environments.

The distribution of euryhaline fish species along salinity gradients provides an opportunity to investigate how osmoregulatory and predatory escape performance influence range limits and distribution patterns. Although the Trinidadian guppy, *Poecilia reticulata,* is considered euryhaline and can be found in fresh, brackish, and marine habitats throughout their geographic range, they are confined to freshwater environments on the island of Trinidad (Magurran 2005; Torres-Dowdall et al. 2013). Several hypotheses have been proposed to explain why *P. reticulata* is confined freshwater, including interactions between salinity and competitors (Torres-Dowdall et al. 2013; Mauro and Ghalambor 2020) and parasites (Robison 2018). In contrast, the closely related, and less studied, swamp guppy (*Poecilia picta*) co-exists with *P. reticulata* in freshwater on the island of Trinidad, and also spans brackish and saltwater environments (Torres-Dowdall et al. 2013). Previous research on *P. reticulata* indicates that predatory history (Ghalambor et al. 2004), temperature, and kinematic water viscosity (Johnson et al. 1998) all affect burst swimming performance. However, the effect of salinity changes on escape performance has received little attention, particularly in the context of considering how salinity might act as a barrier for dispersal in euryhaline fishes (*but see* Handeland et al. 1996 and Collar et al. 2020). Here, we investigated whether escape and osmoregulatory performance along a salinity gradient provides insight into the distribution patterns of the two closely related guppy species in Trinidad. First, we measured how wild-caught individuals responded to a simulated predatory stimulus and quantified the: 1) latency of initiating a response, 2) distance traveled, 3) maximum velocity, and 4) maximum acceleration away from a startling stimulus. Second, we measured second-generation lab reared individuals and quantified the ability of each species to maintain osmotic homeostasis (osmolality) during salinity challenges. We predicted that because *P. reticulata* is confined to freshwater it might exhibit a greater decline in escape performance and have greater difficulty maintaining osmotic homeostasis

with increasing salinity relative to *P. picta* which occupies freshwater and brackish water environments in nature.

METHODS

Animal Collection and Husbandry

We collected adult male *P. picta* (n=60) and *P. reticulata* (n=60) in March 2020 from a freshwater portion of the Caroni River (Lat: 10.6207; Long: -61.4567) in Trinidad where both species coexist. We focused on males, because variation in the stage of pregnancy has been shown to alter swimming performance in females (Ghalambor et al. 2004). Adult males were transported to the field station in Manuel Congo, Trinidad, and individuals (n=20/tank) were placed in conspecific group aquaria containing approximately 15 gallons of freshwater (0 psu). Aquarium water was pre-treated with Kordon AmQuel® Plus, API® Stress Coat, and PraziPro™ to rid the water of unwanted/harmful chemicals and cleanse the fish of parasites. Fish were fed API® Tropical Greens flakes *ad lib* once daily. Individuals were kept in group tanks for at least 72 hours before beginning the experiment to carefully observe and remove any sick or injured fish.

Salinity Acclimation

Acclimations and trials were performed in order of least to highest salinity (0 psu, 6 psu, 9 psu, 12 psu, 15 psu, and 18 psu) so that we tested one salinity per day. A total of 60 males (30 *P. reticulata* and 30 *P. picta*) were used in this experiment. Fish remained in group tanks until 24 hours before their trials began. To test burst swimming performance at different salinities, we sequentially acclimated groups of fish to increasing salinities (see Table 3.S1). Each day, 5 individuals per species were removed and transferred to individual containers consisting of treated

water reflective of their test salinity. Individual removal from group tanks was staggered throughout the day in order to ensure that fish were not acclimating in their test salinity longer than approximately 28 hours before their trials. Once five fish were removed from the stock tank to participate in the experiment the following day, we then transferred the remaining fish to new stock tanks that were pre-treated and set to a new salinity to allow remaining fish to achieve a gradual acclimation up to their specific test salinity. Fish in individual holding containers were fasted during their 24-hour acclimation to ensure that all individuals were in a similar post-absorptive state and then transferred individually to the experimental arena. Individual holding containers were emptied, sanitized with 90% EtOH and rinsed between uses at different salinities so they could be reused the following day.

Experimental Arena and Design

To evaluate burst swimming performance, we placed individual fish into a glass tank with a white 1-cm^2 reference grid placed below. In the center of the grid, a circle (16 cm in diameter) was drawn to provide a guideline for when the fish was centered in the experimental arena. The tank was wrapped in opaque film so that fish could not see the observer or surrounding equipment. Three softbox lights (Mountdog 1350W) were placed around the tank in order to increase lighting while reducing shadows. The tank was divided into three sections using a mesh divider that was impenetrable by the fish but still allowed for water flow. A small heater and pump were placed behind the divider on the right side of a tank to maintain constant temperature. The pump was connected to a tube that led to the leftmost side of the tank, circulating heated water from the right to left sides of the tank. This ensured that there was equal flow of heated water across the two ends and into the center arena, controlling the temperature of the water. The flow, however, was not strong enough to create a noticeable water current within the arena. A thermometer was positioned

inside the leftmost portion of the tank and water temperature was held constant at 30°C for the entirety of each trial in the individual tanks, which is similar to the average temperature from their natural environment. Water depth was kept constant across trials at approximately 8 cm in height to reduce vertical movement of fish (see Figure 3.S1 for experimental apparatus). Each individual completed two trials with at least a 30-minute acclimation period before beginning the first trial, and at least a 10-minute recovery period before beginning the second trial.

To stimulate an escape response, a tennis ball connected to a string and attached to a bar directly above the tank, was swung into the side of the tank. A metal wire attached to the bar at a perpendicular angle determined the angle of the string and tennis ball before release so that it was consistent for each trial. The tennis ball was not released until the fish remained still for several seconds within the marked circle. All trials were filmed at 795.54 frames/s using a Sanstreak SC1 Edgertronic Monochrome high-speed camera. We chose this frame rate because previous work has shown a minimum frame rate of 500 fms/s is necessary to estimate velocity and acceleration (see Walker 1998). The camera was positioned on a tripod directly above the tank at a fixed height that allowed the middle portion of the tank (containing the fish) to be entirely visible in the frame, as well as the impact site of the tennis ball against the experimental tank (see below). Though ripples were created by the tennis ball, video quality was not impaired as the C-starts were typically initiated before the ripples reached the fish. All recorded videos were saved as .mov files.

Five *P. picta* and five *P. reticulata* were tested each day. To remove any order effects, the order of species tested was alternated within and between days. Upon completion of their second trial, fish were immediately dried using a Kimwipe® and weighed to obtain mass. After weighing, fish were placed on white background with a scale and photographed to obtain length measurements. Length measurements were obtained from photos using ImageJ.

Quantifying Reaction Time

Videos were imported into Tracker Video Analysis and Modeling Tool (Version 3; Brown et al. 2021) in order to advance videos one frame at a time. Reaction time (in seconds) was quantified as the number of frames that elapsed between the time that the tennis ball made contact with the side of the tank and the fish first responded (i.e., the first frame where movement was observed) divided by the filming rate (795.54 frames per second). Two observers analyzed each video independently to obtain separate estimates of reaction time, and in instances where discrepancies occurred, the two values were averaged. Each fish participated in two escape trials and thus had two reaction time values, but we used the trial with the quickest reaction time in subsequent statistical analyses.

Quantifying Swimming Velocity and Acceleration

To quantify the kinematics of the escape response of guppies, we digitized and tracked 6 points in 2 dimensions along the midline of each fish during the fast start behavior (see Figure 3.S2) by hand using Tracker Video Analysis and Modeling Tool (Version 3; Brown et al. 2021). Points were tracked from the frame immediately before the first visible movement to the frame with the last visible movement, resulting in tracks that spanned 9-17 frames (mean 14 frames). A custom script in Matlab (R2017b, The Mathworks, Inc., Natick, MA) was used to calculate linear performance variables using the estimated center of mass (CoM) of the fish, following Walker et al. (2005). First, the 6 manually digitized centerline points were interpolated to 50 evenly spaced points such that they were located at distances approximately 0.02-0.04 cm apart along the fish midline. Next, velocity of each point was calculated as distance travelled across successive frames. The point with the minimum sum of velocity values across frames was chosen to represent the CoM (points 13-18). To reduce the variation in CoM position as a result of variation across

successive interpolated midlines, the chosen point was then smoothed using a quintic spline. Tolerance values were manually adjusted to maximize the fit to the original kinematic trace while also reducing large spikes in derived values. Smoothed CoM position was then used to determine net linear distance traveled ($d_{net(t)}$), maximum velocity ($V_{max(t)}$), and maximum acceleration ($A_{max(t)}$) within the first 5 and 10 ms (t), which corresponded to frames 5 and 9. Fish standard length was calculated as the maximum linear distance between the manually digitized head (point 1) and tail (point 6) locations.

Quantifying Osmolality

In the lab at Colorado State University, we used second generation (F2) lab born fish to quantify how the acclimation scheme outlined above affects internal osmolality for each species. Based on availability, females were used for both *P. reticulata* and *P. picta*. These fish were originally born and reared in 30 psu. All fish were transferred to 15 psu aquaria where they remained for 4 days prior to being transferred to aquaria containing freshwater (0 psu). All fish remained in freshwater for 14 days to allow for proper restructuring of gills to obtain gills similar to those individuals reared in freshwater (Brennan et al. 2015). After this freshwater acclimation period, the total number of fish per species were split equally into 6 groups (~3-5 individuals) and then transferred into separate 2.5-gallon aquaria. After 2 days, 3 *P. reticulata* females in one freshwater aquarium and 3 *P. picta* females from another freshwater aquarium were euthanized in order to obtain blood plasma following the procedure outlined below. All individuals in other aquaria were subjected to the same salinity acclimation increase that wild-caught fish were subjected to.

Blood plasma was extracted following methods described in Havird et al. (2014) to increase plasma extraction volume compared to other conventional blood extraction methods (e.g., syringe,

caudal peduncle clipping, etc.). Specifically, fish were transferred to a dry, non-porous surface and the exterior of each individual was gently dried with a Kimwipe®. Once dried, each fish was decapitated using a straight-edge blade, and their brains were pithed to ensure rapid euthanasia. Following decapitation, fish were immediately transferred (exposed side down with caudal fin facing upward) into a 0.22um centrifuge filter tube and spun down for 12 min at 13,400 rpm. Limiting the time between decapitation and the centrifuge process reduced blood coagulation. Using a filter tube allowed tissue to separate from fluids to ensure that fluids were not contaminated. The filters and fish remains were removed from the tube, and tubes containing internal fluids were then transferred to -20˚C prior to osmolality quantification.

After the experiment was complete, samples were defrosted and centrifuged for 5 minutes at 13,400 rpm to separate plasma from any red blood cells. 2uL of plasma from each sample was transferred via a 2uL-10uL pipette to a Wescor Vapor Pressure Osmometer to quantify osmolality (mmol/kg).

Data archival

All analyzed videos were uploaded to ZMAportal.org (Brainerd et al. 2017).

Statistical analysis

Mass, Length, and Body Condition. For each individual in the study, we measured mass (g), length (cm), and calculated body condition (mass/length^3) using Fulton's condition factor (Neumann et al. 2012) as possible covariates of the performance metrics (i.e., velocity, acceleration, net distance traveled, and reaction time). Mean values were also recorded for each salinity (Table 3.S2). As both the covariates and response variables were potentially correlated with each other, we used a multivariate general linear model that included all the covariates as

predictor variables and all the performance metrics as the response variables. However, none of the potential covariates were significant in either of the two species (*P. picta* $p > 0.751$; *P. reticulata* $p > 0.19$). To investigate the relationship further, we 1) extracted the first principal component from the multivariate analysis for performance metrics and plotted it against body condition to visually inspect the relationship (Figure 3.1) and 2) regressed all three measures of size against the performance metrics for each species (Table 3.1). In no case was size a significant predictor of performance. Thus, we dropped size from subsequent tests examining the effects of species and salinity on performance.

Escape Performance. Because the four parameters of the escape response were correlated with each other, a MANOVA was conducted that included the fixed effect of species (*P. reticulata* vs. *P. picta*), the test salinity (0 psu, 6 psu, 9 psu, 12 psu, 15 psu, and 18 psu), and their interaction. Principal components were extracted for each individual to visualize the combined measure of performance as a function of salinity using the `prcomp()` function in R.

To aid in the interpretation of the multivariate results, we then ran separate ANOVAs on each of the univariate scores. Estimated marginal means were then calculated using the `emmeans` package in R to determine how reaction time, distance traveled, velocity, and acceleration each contributed to the multivariate result. Tukey adjusted pairwise comparisons between the six salinity treatments for each species were conducted using the `pairs()` function in R.

Osmolality. Measurements were recorded for 2-4 female individuals for each of the experimental salinities (see Table 3.S3). We used a linear model to determine the relationship between test salinity and osmolality with both *P. reticulata* and *P. picta* partitioned out in the model. The `emmeans` package in R was used to generate estimated marginal means for osmolality values for each species and salinity treatment (Table 3.S3). Tukey adjusted pairwise comparisons

between the six salinity treatments for each species were conducted using the `pairs()` function in R.

RESULTS

Multivariate Analysis of Escape Performance

The multivariate response revealed that salinity was a marginally significant predictor of escape performance (Wilk's Lambda = 0.469, $F_{20,41}$= 1.63, p = .055), whereas species (Wilk's Lambda = 0.855, $F_{4,41}$= 1.61, p = .191), and the interaction between species and salinity were not (Wilk's Lambda = 0.684, $F_{20,41}$=0.77, p = .743) (Figure 3.2). The marginally significant result in salinity is driven by a decrease in escape performance in both species after being transferred from 0 psu to 6 psu (Figure 3.2). At 9 psu, the performance of *P. picta* recovered, but *P. reticulata* performance did not recover until 12 psu (Figure 3.2). Examination of the univariate results revealed that the decrease in the composite measure of performance at 6 psu is most strongly driven by a drop in acceleration, but also decreased velocity, distance travelled, and slower reaction times (Figure 3.3).

Osmolality

Osmolality was measured in 2-4 female individuals of each species for the six different salinity treatments. Tukey adjusted pairwise comparisons revealed that there were no intraspecific differences in osmolality between any of the salinity treatments. However, there was a significant difference between species at 0 psu with a higher osmolality for *P. picta* than *P. reticulata* (p = .048) (Figure 3.4).

DISCUSSION

Performance curves are useful to predict how species might respond when subjected to changes in their external environment (Huey and Stevenson 1979; Sinclair et al. 2016, Huey and Kingsolver 1989). For example, in the face of global climate change, it is expected that low lying areas will experience upstream infiltration of brackish water and saltwater, thus affecting the ecology and distributional patterns of fish species (Gehrke et al. 2011). Salinity performance curves (SPC's) can allow for a better understanding of species' sensitivities to increased salinity, providing insight into how species will fare in the future. Previous work has shown that faster responses and longer distances traveled lead to more successful evasion of predators (Walker et al. 2005), confirming that SPC's for escape performance are likely to be correlated with fitness. Here, we generated SPC's to investigate how increasing salinity influences aspects of escape performance and plasma osmolality in two closely related species of euryhaline fishes that differ in their distribution patterns on the island of Trinidad. Specifically, we wanted to test the hypothesis that *P. reticulata* exhibits reduced performance in brackish water and this contributes to their restricted distribution in freshwater. In contrast, we predicted because *P. picta* occupies both fresh and brackish water, we predicted it should exhibit similar performance across the salinity gradient.

Overall, when we looked at the multivariate escape response we found a marginally significant effect of salinity on escape performance, where both species exhibit an initial decrease in performance when transferred from 0 psu to 6 psu (Figure 3.2). However, although the species by salinity interaction term was not significant, escape performance recovered more quickly from 6 psu to 9 psu in *P. picta* compared *P. reticulata* (Figure 3.2). Indeed, the only salinity where both species exhibited significant differences in performance was 9 psu ($F = 13.44$, $p = 0.014$). This same pattern is seen in the univariate results, particularly for maximum velocity and reaction time,

and to a lesser degree in acceleration (Figure 3.3). Together, these results suggest that *P. picta* can acclimate more quickly to a salinity challenge compared to *P. reticulata*. A slower acclimation response in *P. reticulata* could be a contributing factor to its avoidance of brackish water because of the initial reduction in vulnerability to predation. However, a critical caveat to this work is that our conclusions were based solely on escape performance measurements in male *P. reticulata* and *P. picta*. Without having measured the performance of females, we cannot say for certain that females would show a similar escape pattern.

There are many factors, both biotic and abiotic, that can influence escape performance in a fish's native environment. All four parameters measured in this current study are known to influence predator evasion success in fish, but the difficult nature of measuring the consequences of poor escape performance and reaction time has led to a lack of representation in the literature over the years (Domenici and Hale 2019; Walker et al. 2005). However, previous work in guppies has revealed that even a slight (one standard deviation) increase in escape performance can lead to a successful evasion versus a capture (Walker et al. 2005). Using wild-caught fish, we were able to capture a more ecologically relevant response in the face of increasing salinity exposure for both species. While not historically considered to be schooling fish, both species tend to be found in conspecific groups. In fact, in lowland freshwater streams on the island of Trinidad, these species are even known to shoal together. Investigations into the potential environmental influences on escape performance have shown that social groups have positive impacts on reactivity to stimuli (Nadler et al. 2021). Thus, we might expect slight differences in reaction time for fish in groups, but we would not expect differences in velocity, acceleration, or distance traveled. Among other factors that may influence escape performance, previous work in Hawaiian stream gobies *(Sicyopterus stimpsoni*) revealed that the intensity of currents in flowing streams can sometimes

mask environmental stimuli, leading to disturbances in the lateral line detecting the stimulus from a predator (Diamond et al. 2016).

The ability to osmoregulate and maintain homeostasis is important for biological processes and survival (Kültz 2015), and maintaining consistent blood plasma osmolality in the face of a salinity challenge is indicative of possessing enhanced osmoregulatory mechanisms. While we found differences in the speed of salinity acclimation between the two species, we were unable to collect blood osmolality data on these individuals. To further explore differences in the ability to maintain homeostasis, we used *P. picta* that were both born and reared in saltwater (30 psu) and transferred down to freshwater prior to being subjected to the same experimental salinity increase used during the burst swimming experiments in the field. Both species were capable of rapidly acclimating down to freshwater from saltwater and no mortalities were recorded (Figure 3.4). In fact, mortalities were not observed for either species during the 14-day freshwater acclimation period nor following the salinity increase up to 18 psu. Although no mortalities were recorded, we cannot explicitly say that one species was more challenged, or expended more energy to maintain homeostasis, than the other. However, interspecific differences were observed between *P. reticulata* and *P. picta* in freshwater as *P. reticulata* exhibited significantly lower plasma osmolality than *P. picta*. The ability of both species to maintain homeostasis during a salinity acclimation was evidence to support true euryhalinity, similar to what has been found in other species, including the closely related sailfin molly (*Poecilia latipinna*) (Nordlie et al. 1992). However, intraspecific variation between freshwater and saltwater has been observed in many other teleost species (Nordlie 2009). For example, ninespine stickleback (*Pungitius pungitius*) show substantial increases in osmolality in saltwater versus freshwater, whereas threespine stickleback (*Gasterosteus aculeatus*) exhibit minimal difference in internal osmolality in

freshwater and saltwater (Nordlie 2009). Thus, the degree to which osmolality is maintained in differing salinity environments is not universal for all euryhaline fishes.

Domenici and colleagues (2019) suggest that experiments should include interacting effects of multiple stressors that fish populations might experience in the face of global climate change, each with at least three treatment levels. Thus, future work could incorporate the interacting effects of salinity with increasing temperatures. Although we do not expect temperature changes to significantly affect osmoregulatory capacity in either species, temperature increases have been shown to affect bone deposition and remodeling over ontogeny in other fish species (Campbell et al. 2021). Thus, it is possible that functional morphology will be broadly impacted by climate change (Mabee et al. 2000; Ramler et al. 2014), and since changes in morphology and bone structure can affect swimming performance, escape performance may also be negatively impacted.

Conclusion. In their natural environments on the island of Trinidad *P. reticulata* is restricted to freshwater, while *P. picta* occupies the full range of salinity from freshwater to seawater. Here, we find support for both species being physiologically euryhaline, but *P. reticulata* appears to have a slower acclimation response, as *P. picta* recovers more quickly from a salinity challenge. Thus, salinity alone does not appear to be acting as a barrier to dispersal in *P. reticulata*, but if a slower acclimation response results in increased predation, then predators could indirectly be contributing to the range limit. Future work could test this hypothesis more directly by examining survival of *P. reticulata* in the presence of predators (similar to Walker et al. 2005) and testing if survival increases as a function of different acclimation times.

TABLES & FIGURES

Table 3.1. *F* and *p* values from an ANCOVA for mass, length, and body condition on acceleration, velocity, net distance traveled, and reaction time for *P. reticulata* and *P. picta*.

Escape Performance	Species	Measurements	*F*	*p*
Reaction Time	*P. reticulata*	Mass	0.00	.953
		Length	0.94	.345
		Body Condition	0.48	.498
	P. picta	Mass	0.63	.436
		Length	0.14	.713
		Body Condition	1.15	.293
Net Distance Traveled	*P. reticulata*	Mass	0.00	.977
		Length	0.07	.796
		Body Condition	0.05	.830
	P. picta	Mass	0.65	.428
		Length	0.02	.885
		Body Condition	3.38	.080
Velocity	*P. reticulata*	Mass	0.73	.404
		Length	3.34	.083
		Body Condition	0.47	.502
	P. picta	Mass	0.00	.995
		Length	0.39	.542
		Body Condition	0.42	.521
Acceleration	*P. reticulata*	Mass	0.20	.657
		Length	0.58	.457
		Body Condition	0.02	.890
	P. picta	Mass	0.56	.464
		Length	1.97	.176
		Body Condition	0.36	.556

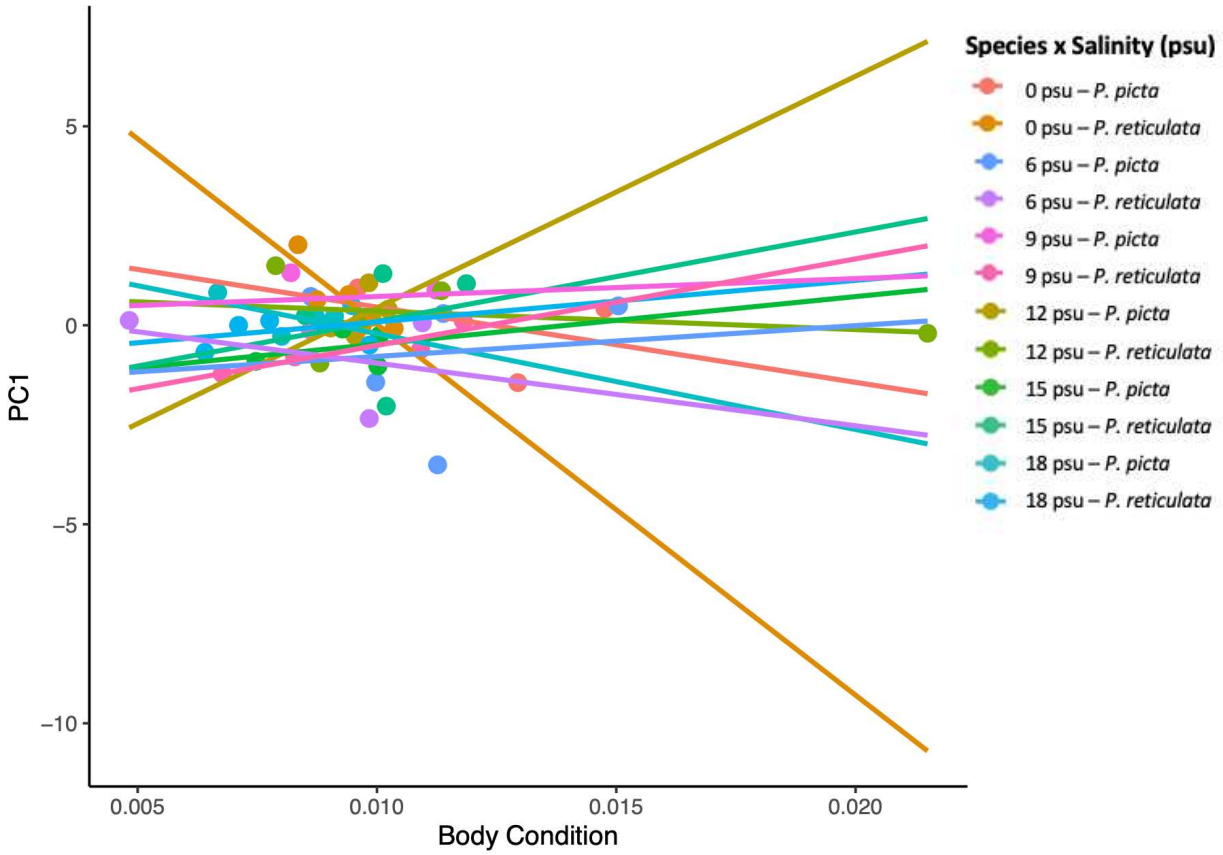

Figure 3.1. Depiction of relationship between PC1 values and body condition (mass(g)/length(cm)^3) for each individual. Colors denote the specific salinity treatment and species of the individual. Regression lines for each salinity treatment per species are represented.

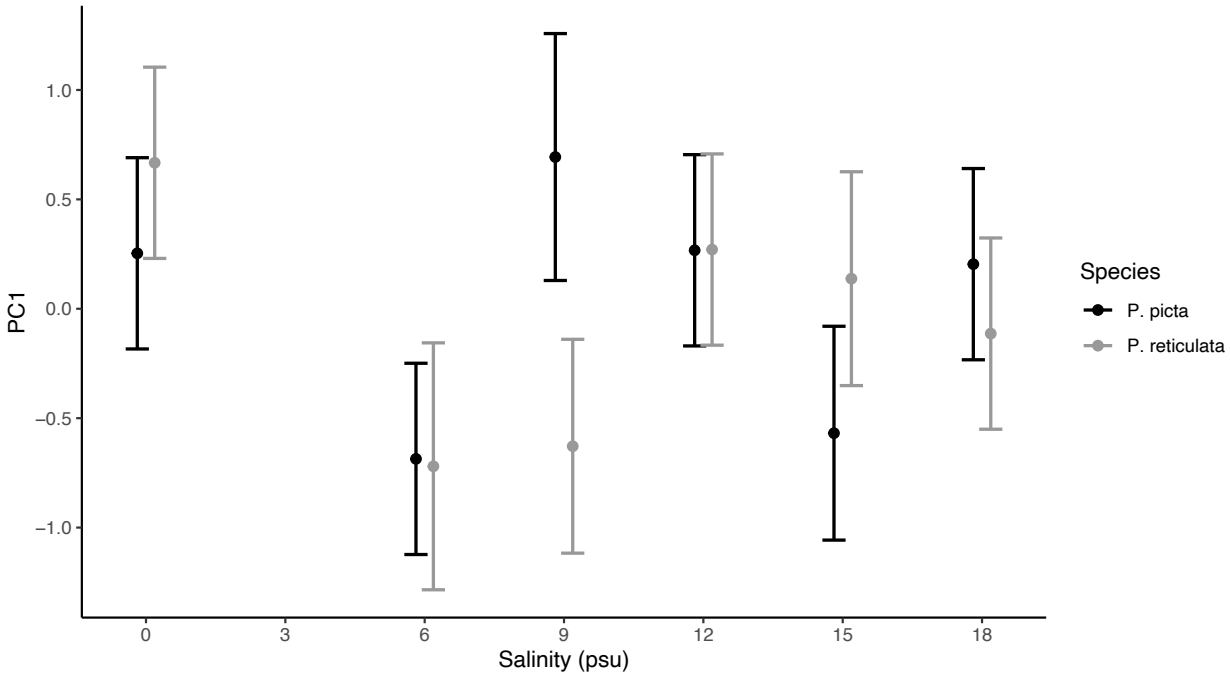

Figure 3.2. Estimated marginal means (EMMean ± SE) for escape performance generated from individual PC1 scores at each test salinity.

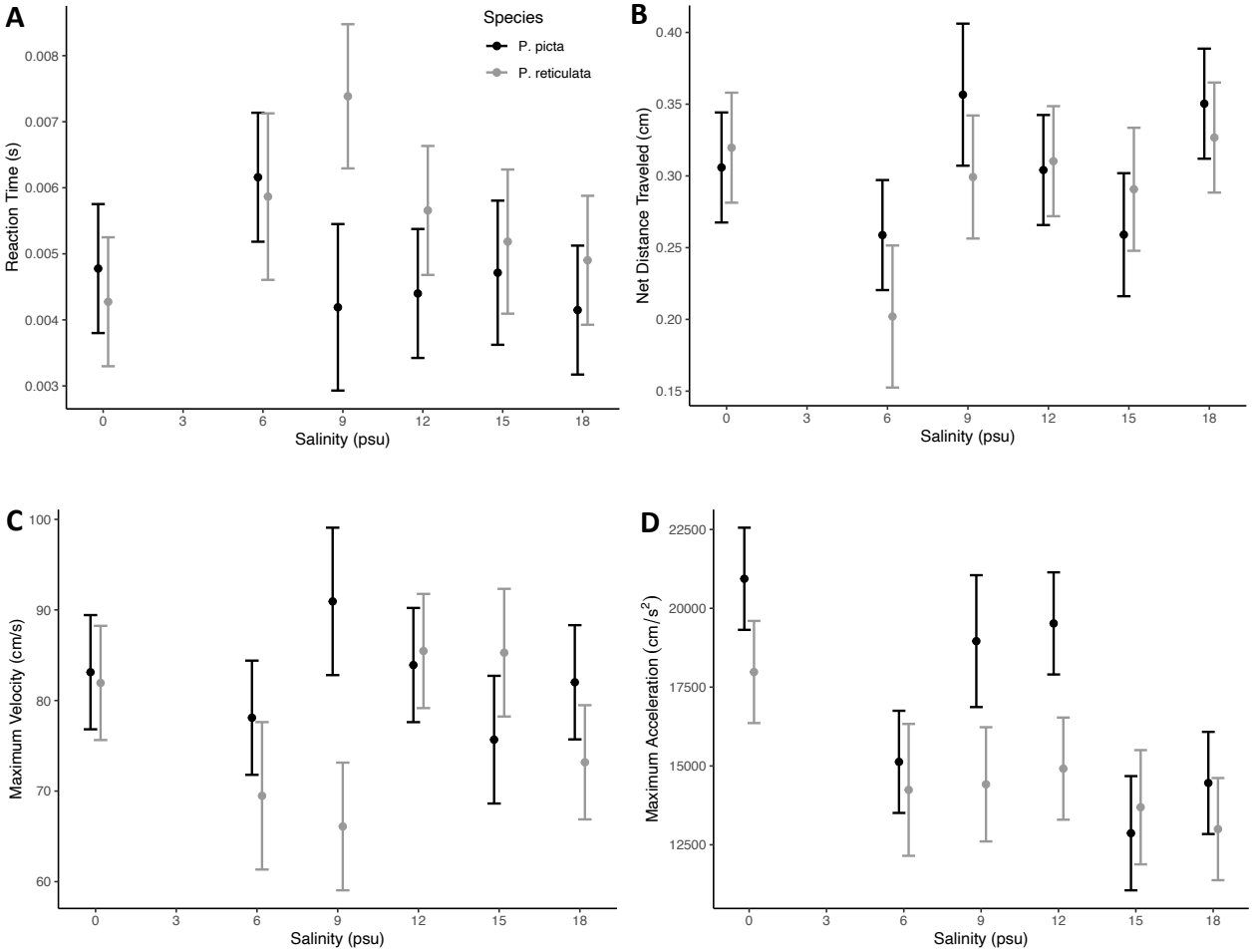

Figure 3.3. Depiction of the relationship between ambient salinity and univariate escape responses for (A) reaction time, (B) net distance traveled, (C) velocity, and (D) acceleration (EMMean ± SE) in both *P. reticulata* (gray circles) and *P. picta* (black circles).

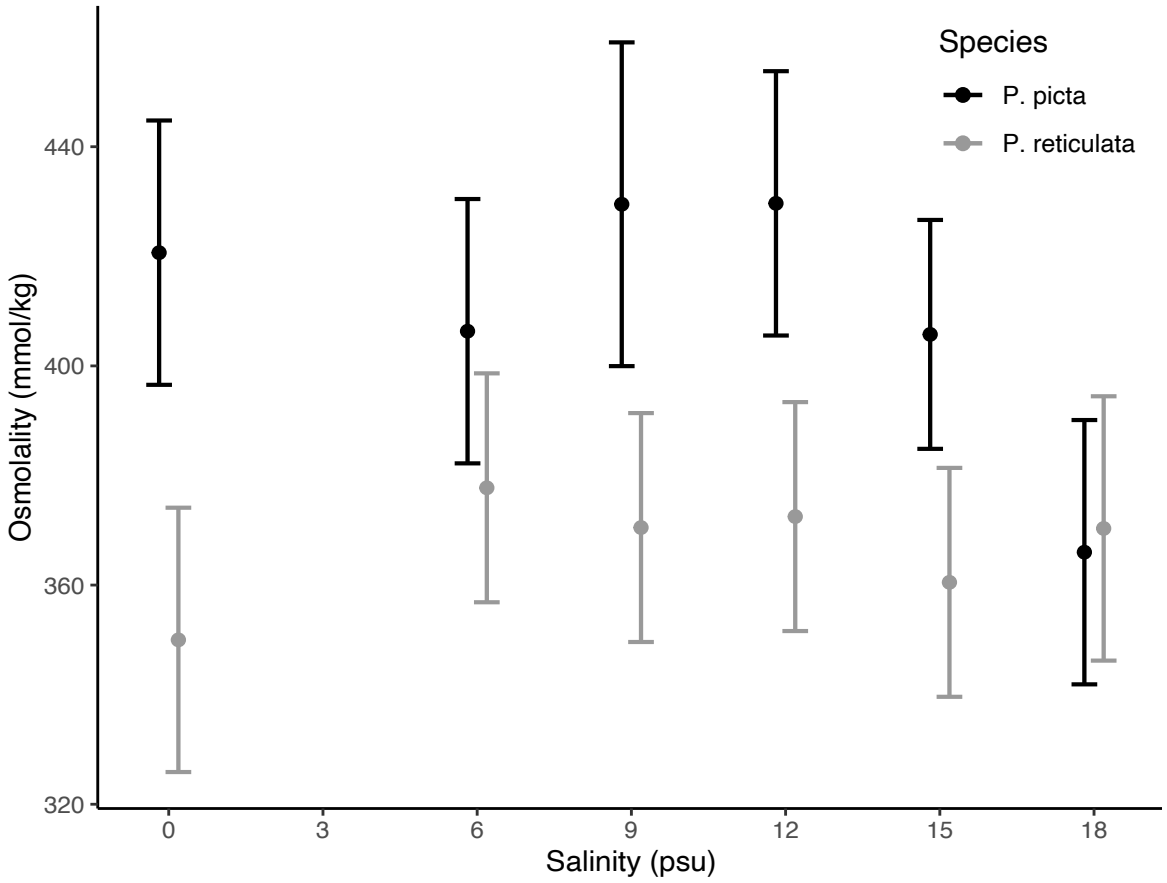

Figure 3.4. Depiction of the relationship between ambient salinity and plasma osmolality (circles, EMMean ± SE) in female *P. picta* (black circles) and female *P. reticulata* (gray circles) subjected to increasing salinity treatments.

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CHAPTER 4: QUANTIFYING CORTISOL CONCENTRATIONS IN NATURAL POPULATIONS OF A EURYHALINE FISH ALONG A SALINITY GRADIENT: A NOVEL METHOD REVEALS ELEVATED CORTISOL IN RESPONSE TO VARIABLE SALINITY LEVELS

INTRODUCTION

A fundamental goal in eco-physiology is to understand how physiological processes contribute to distributional patterns in nature (Irschick and Garland 2001; Irschick et al. 2008). Because environmental heterogeneity can contribute to divergence at local scales, the effect of environmental influences on population structure and dispersal potential has received considerable attention in recent years (Schluter 2000; Nosil 2012). Investigating the effects of environmental influences on physiological mechanisms can provide insight into distribution patterns in nature and the potential for dispersal to novel environments.

For aquatic species, local and global distribution patterns have been shown to be significantly influenced by the physiological challenges imposed by different salinity levels (Kefford et al. 2004; Kefford et al. 2012; Lisboa et al. 2015). Even low levels of salinity can be a major barrier to dispersal for freshwater species, and saltwater adapted marine species typically avoid freshwater and low salinity environments (Ern et al. 2014). Such patterns arise because of the contrasting mechanisms used to osmoregulate in a given salinity environment (Hochachka and Somero 2002; Evans et al. 2005; Hwang and Lee 2007). These osmoregulatory mechanisms have been studied extensively in euryhaline fishes because they exhibit plasticity in their ability to osmoregulate under different salinities, allowing them to occupy the full range of aquatic environments from freshwater to saltwater (Schultz and McCormick 2012). In freshwater, gills absorb ions from the external environment whereas in saltwater, they aid in secreting excess salt ions back into the environment (Marshall and Grosell 2006; Foskett et al. 1981; Evans et al. 2005). Freshwater gills

are comprised of mostly pavement, respiratory, and chloride cells, also known as ionocytes. These cells are also present in saltwater acclimated gills; however, the addition of accessory cells create leaky junctions adjacent to ionocytes, allowing for paracellular elimination of Na^+ ions following Cl^- secretion (Laurent 1984; Evans et al. 2005). Excess salt ion secretion is aided by three ion transporters in saltwater ionocytes: Na^+K^+-ATPase (NKA), $Na^+K^+2Cl^-$ cotransporters (NKCC), and cystic fibrosis transmembrane conductance regulators (CFTR) (Hiroi et al. 2005; Hiroi et al. 2008). In addition, increases in salinity typically result in changes of ionocyte location, with ionocytes typically exhibiting localization in the filament, but expanding to the lamellae following salinity increase (Evans et al. 2005) as well as changes in ionocyte density and size (Kultz et al. 1992, Kultz et al. 1995; Hiroi et al. 2005; Evans et al. 2005).

The plasticity of osmoregulatory mechanisms exhibited by euryhaline fish is controlled by the endocrine system (Hiroi and McCormick 2012; Mackie et al. 2007; McCormick 2001; Pelis and McCormick 2001; McCormick and Bradshaw 2006). In euryhaline fish, a diversity of hormones have been shown to be involved in osmoregulation, including arginine vasotocin (AVT), cortisol, growth hormone, prolactin, and insulin like growth factor (McCormick and Bradshaw 2006; Sakamoto and McCormick 2006; McCormick 2011; Mancera and McCormick 2019). These hormones work together across different tissues over a continuum of temporal scales to maintain ion homeostasis. However, in response to a salinity challenge, different sets of hormones act during the initial “acute phase” when existing ion transport mechanisms are activated, versus the “acclimation phase” when new cells and proteins are developed (McCormick and Bradshaw 2006). A large body of research has shown that cortisol plays a key role during both the acute and acclimation phases to promote salinity tolerance (Mancera and McCormick 2019). Although cortisol was originally known to promote survival and osmoregulation when fish acclimate to

saltwater (*reviewed by* McCormick 1995 and Mommsen et al. 1999), studies on ion uptake in freshwater trout (*Salmo gairdneri*) have shown that cortisol is also involved in increased Na^+ and Cl^- uptake for fishes living in freshwater environments (Laurent and Perry 1990; Lin and Randall 1993). In euryhaline fishes, a salinity challenge that causes changes in internal osmolality triggers the release of cortisol from the Hypothalamus-Pituitary-Interrenal (HPI) axis into the blood plasma, which in turn increases expression of NKA and NKCC in the gills, and over longer time periods leads to proper gill modification and successful acclimation (Mancera and McCormick 2019; McCormick 2011; Tort 2011). For example, previous work in tilapia (*Oreochromis mossambicus*) has shown that upon experiencing a salinity change, cortisol levels increase rapidly and peak after 3 days, after which there is a return in plasma osmolality and cortisol levels begin to decrease (Kammerer et al. 2010). This pattern of an increase in cortisol followed by a return to pre-salinity challenge levels is a general pattern observed across a wide diversity of euryhaline fishes exposed to moderate changes in salinity (e.g. Mancera et al. 1994; Madsen et al. 1996; Marshall et al. 1999; Tsui et al. 2012). Thus, under stable salinity conditions, a fully acclimated fish should possess baseline cortisol levels, whereas under fluctuating salinity cortisol levels are expected to be elevated. Not surprisingly, most of our current knowledge regarding the role of cortisol in osmoregulation is primarily from experiments that have been conducted in a lab setting, however few studies have measured how natural populations of euryhaline fish cope with fluctuating or stable salinity conditions. One reason few studies have measured natural populations is because of the logistic challenges of rapidly collecting, preparing, and assaying samples for cortisol. In this study, we use a novel methodology of extracting cortisol from ethanol preserved fish to test how cortisol levels vary between sites with stable or fluctuating salinity.

On the island of Trinidad, *Poecilia picta*, a relatively understudied euryhaline fish species, spans the salinity gradient from freshwater to brackish and marine environments (Torres-Dowdall et al. 2013). Thus, at one end of the gradient *P. picta* experience constantly high salinities that are likely well above the isosmotic point, while at the other end of the gradient they only experience freshwater (Torres-Dowdall et al. 2013). Between these two extremes, *P. picta* experience fluctuating salinities largely because of daily tidal influences. We predicted circulating cortisol levels should be elevated under more variable salinity conditions and lower under more stable salinities. To test this prediction, we compared free, whole-body cortisol levels for ethanol-preserved *P. picta* found in adjacent freshwater, brackish, and marine habitats on the island of Trinidad. Extracting cortisol from fish preserved in ethanol is beneficial for rapidly capturing cortisol levels with minimal equipment, which is advantageous when there is a long time between collecting samples in the field and when they can be assayed in the lab, as was done in this study.

Here, we focused on contrasting free cortisol of fish in environments with stable salinity unaffected by tidal fluctuations, including upstream freshwater sites (always 0 psu) and coastal marine sites (20+ psu), versus variable brackish environments subject to daily fluctuations in salinity (1-19 psu). By sampling and comparing natural populations of *P. picta* along salinity gradients in three distinct rivers (Caroni, Caparo, and Cunupia) we were able to test the effects of tidal fluctuations on free cortisol. We hypothesized that fish would be fully acclimatized to stable salinity environments (0 psu and 20+ psu) and should have comparably lower concentrations of cortisol compared to fish experiencing fluctuations in salinity above and below the isosmotic point (1-19 psu) (Figure 4.1A). However, if cortisol changes quickly in response to salinity, reverting to baseline levels, we might not observe differences across the gradient (Figure 4.1B). Conclusions were validated through quantifying the effect of salinity fluctuation on free cortisol levels in a lab

setting by subjecting fish to a brackish water titration (0 psu to 15 psu) versus a freshwater titration control (0 psu to 0 psu). An additional validation for the novel method was conducted by comparing cortisol levels for stressed (i.e., chased) fish versus non-stressed fish at rest.

METHODS

Animal Collection

Approximately 10 male and 10 female *P. picta* were collected from 4 differing salinity ranges: stable freshwater (0 psu), variable brackish low (1-9 psu), variable brackish high (10-19 psu), and stable brackish/saltwater (20+ psu) in the Caroni, Cunupia, and Caparo Rivers on the eastern coast of Trinidad (N=240). The variation in salinity at each of these sites was established based on repeated sampling on different days. Fish were gently captured from their natural environment via butterfly nets. Care was taken to ensure slow and steady movements through the water during capture to minimize stress. Fish were immediately transferred to an ice bath and then removed once visually euthanized (as determined by a lack of gill movement). Upon removal from the bath, excess water was removed from the exterior of the fish using a Kimwipe™ and individuals were added to a 40mL glass vial (~4x the size of the fish) containing 95% ethanol (Figure 4.S1).

Quantification of Free Cortisol from 95% EtOH

After at least 2 months, the fish were removed from the EtOH and weighed to determine mass. Quantification of free cortisol from fish preserved in 95% EtOH was conducted using a validated novel method (see *Stress-induced method validation* below). Ethanol from the glass vial was poured into a 600 mL beaker (pre-cleaned with EtOH and distilled water, then dried). 240 mL (~6x the amount of EtOH) of distilled water was added to the beaker. Labeled C18 Cartridges

(ThermoScientific™ HyperSep™) were activated in order to collect free cortisol from the EtOH samples. To activate, C18 Cartridges were placed on a vacuum manifold rack and 2 mL of 100% methanol (HPLC grade) were pipetted into each cartridge. The vacuum pump was then turned on to draw the methanol through each of the cartridges. An additional 2 mL of 100% methanol was pipetted into the cartridges followed by another round of vacuuming. With the pump off, 2 mL of distilled water was pipetted into each cartridge followed by initiation of the vacuum pump for a brief period of time, allowing some distilled water to remain in each cartridge to prevent the cartridge from drying out.

To extract cortisol from the EtOH in the beaker, diluted EtOH was run through a C18 Cartridge, trapping free and conjugate cortisol. Using Tygon® tubing (pre-cleaned internally and externally with EtOH) wrapped with parafilm on one end to increase suction over the cartridges, diluted EtOH was vacuumed over the corresponding cartridge until the beaker was completely empty. Once all samples were run through the cartridges, 2 mL of distilled water was pipetted into each cartridge and vacuumed down to remove excess salts.

Labeled 13 x 100 mm borosilicate glass vials were placed in a vacuum manifold rack underneath the corresponding C18 Cartridge. To collect free cortisol only, 2 mL of ethyl acetate (HPLC grade) was pipetted into each C18 Cartridge. The vacuum pump was then turned on to run the ethyl acetate through, collecting all liquid into the borosilicate vials below. This procedure was repeated with another 2 mL of ethyl acetate to collect all free cortisol trapped in the cartridges. C18 Cartridges were then discarded.

Borosilicate vials containing free cortisol and ethyl acetate were placed into a 37°C water bath along with an evaporating manifold connected to a nitrogen tank. Nitrogen was carefully flowed into the vial to dry out the liquid sample, leaving only dried free cortisol. Cortisol was then

resuspended using 0.1M phosphate buffer, covered with Parafilm® and aluminum foil, and stored in -20°C prior to ELISA assay. Free cortisol was then quantified using an ELISA assay (Cortisol ELISA Kit - Cayman Chemical).

Validation of salinity's effects on cortisol

In order to establish a correlation between the effect of salinity fluctuation on cortisol, we conducted a validation using a salinity titration vs. a control. For this method, 24 male and 24 female *P. picta* (N=48) were collected from a freshwater (0 psu) portion of the Caroni River (Lat: 10.6207; Long: -61.4567) in Trinidad and brought back to the field station. Fish were divided equally into two 5-gallon aquaria containing 10,500 mL of water that had been pre-treated with Kordon AmQuel® Plus, API® Stress Coat, and PraziPro™ to rid the water of unwanted/harmful chemicals and cleanse the fish of parasites. Each aquarium consisted of 12 males and 12 females. Fish were fed API® Tropical Greens flakes *ad lib* once daily until 24 hours before the experiment began to ensure fish were in a post-absorptive state. Fish were left to acclimate for a period of 48 hours prior to the experiment. After a period of acclimation, one aquarium was titrated with a high concentration of artificial saltwater (500 mL of 60 psu) made from Instant Ocean® Sea Salt in order to increase the salinity of the container by ~2.5 psu every 15 minutes until a salinity level of 15 psu was reached (Figure 4.S2). Fifteen minutes after the last titration, fish were carefully removed from the aquarium via a dip net, euthanized in an ice bath, and placed in a 40mL glass vial containing 95% EtOH.

The second aquarium acted as a control where 500 mL of freshwater (0 psu) was added every 15 minutes, mimicking the titration of the treatment group to account for any stress that may have been induced by the addition of water. Fifteen minutes after the last titration, fish were

euthanized and preserved using the same method outlined above. At least 2 months after fish were placed in EtOH, differences in free cortisol concentrations were quantified using the cortisol extraction method outlined above.

Stress-induced method validation

To further validate this novel method, we investigated the impact of stress on circulating cortisol. Cortisol is known to be elevated following acute exposure to stressful stimuli (e.g. Mommsen 1999; Ramsay et al. 2009), thus we conducted an experiment where we compared the cortisol levels of stressed and non-stressed fish. We collected 24 male and 22 female *P. picta* from the Caroni River in Trinidad (Lat: 10.6207; Long: -61.4567) and transferred them back to the field station. Fish were housed in two 20-gallon freshwater (0 psu) aquaria. Aquarium water was pre-treated with Kordon AmQuel® Plus, API® Stress Coat, and PraziPro™ to rid the water of unwanted/harmful chemicals and cleanse the fish of parasites. Fish were fed API® Tropical Greens flakes ad lib once daily until 24 hours before the experiment began to ensure fish were in a post-absorptive state. Twelve males and 12 females were subjected to a "non-stressed" protocol while 12 males and 10 females were subjected to a "stressed" protocol (adapted from Ramsay et al. 2009). Non-stressed fish were transferred to a 5-gallon aquarium 24 hours prior to the start of the experiment. After the 24-hour acclimation, fish were removed from the tank, transferred to an ice bath for euthanasia, dried with a Kimwipe™ and placed in a 40 mL glass vial containing 95% ethanol. To ensure minimal stress caused by the observer, an opaque barrier was used, and the net was handled slowly and gently. Fish subjected to the stressed treatment were transferred to a 5-gallon aquarium 24 hours prior to the start of a net-chasing treatment. Following the 24-hour acclimation period, fish were chased around the tank with a net for 3 minutes, followed by a rest

period of 3 minutes, then were subjected to another round of net-chasing for 3 minutes. 15 minutes after the second round of net-chasing, fish were euthanized via an ice bath and transferred to a 40 mL glass vial containing 95% ethanol. All fish from both treatments remained in 95% EtOH for at least 2 months prior to cortisol analysis.

Statistical Analysis

Field Cortisol. To meet assumptions of normality, we used natural log (LN) transformed values of dilution-adjusted cortisol. Because females had a significantly higher mass than males ($F_{1,226}$ = 76.11, p<.001), we regressed the natural log (LN) of dilution adjusted cortisol (pg/sample) against the mass to obtain the residuals of the regression. The residuals were then analyzed using a linear model that incorporated sex (male and female), river (Caroni, Caparo, and Cunupia Rivers), and salinity category (freshwater, low salinity, mid salinity, and high salinity). The `emmeans` package in R was used to generate estimated marginal means for the cortisol residuals for each of the salinity levels. Tukey adjusted pairwise comparisons between the four salinity levels were conducted using the `pairs()` function in R.

Salinity Titration. To meet assumptions of normality, we used natural log (LN) transformed values of dilution-adjusted cortisol. Because females in this experiment had a significantly higher mass than males ($F_{1,45}$ = 22.37, p<.001), we again regressed the natural log (LN) of dilution adjusted cortisol (pg/sample) against the mass to obtain the residuals of the regression. The residuals were then analyzed using a linear model that incorporated sex (male and female) and treatment (freshwater and brackish water titration). The `emmeans` package in R was used to generate estimated marginal means for the cortisol residuals for each treatment. Tukey adjusted

pairwise comparisons between the two treatments were conducted using the `pairs()` function in R.

Method Validation. To meet assumptions of normality, we used natural log (LN) transformed values of dilution-adjusted cortisol. Because females have a significantly higher mass than males ($F_{1,44}$ = 28.11, p<.001), we again regressed the natural log (LN) of dilution adjusted cortisol (pg/sample) against the mass to obtain the residuals of the regression. The residuals were then analyzed using a linear model that incorporated sex (male and female) and treatment (stressed and non-stressed). The `emmeans` package in R was used to generate estimated marginal means for the cortisol residuals for each treatment. Tukey adjusted pairwise comparisons between the two treatments were conducted using the `pairs()` function in R.

RESULTS

Field Cortisol

An ANCOVA revealed that river ($F_{2,221}$ = 27.84, p<.001) and salinity type ($F_{3,221}$ = 8.23, p<.001) were significant predictors of cortisol level. Sex was not a significant predictor ($F_{1,221}$ = 0.33, p = .566); however, conclusions about the effect of salinity level on cortisol were similar regardless of sex being included. Pairwise comparisons between the four salinity levels revealed that cortisol levels in freshwater and high salinity (20+ psu) were not significantly different from one another (p>.05). Cortisol levels in the low (1-9 psu) and mid (10-19 psu) salinities were also not significantly different from one another (p>.05). However, the cortisol levels from fish collected in freshwater were significantly lower than both the low (p<.001) and mid (p = .003) salinities, while the cortisol levels high salinity level were also significantly lower than both the low (p = .002) and mid (p = .02) salinities (Figure 2). Together, these results show that *P. picta* in

freshwater and high salinities have significantly lower cortisol levels than those from low and mid salinities on the island of Trinidad.

Salinity Titration

An ANCOVA revealed that treatment ($F_{1,44} = 18.33$, $p<.001$) was a significant predictor of cortisol levels, and sex was not ($F_{1,44} = 2.54$, $p = .118$). Fish exposed to the brackish water titration had significantly higher cortisol levels (Figure 4.3), and this result was upheld regardless of whether sex was retained in the model or not.

Stress Test Validation

An ANCOVA revealed that treatment ($F_{1,43} = 276.60$, $p<.001$) was a significant predictor of cortisol levels, and sex was not significant ($F_{1,43} = 0.01$, $p = .933$). Stressed fish exhibited higher cortisol levels than non-stressed fish (Figure 4), with similar conclusions regardless of sex being included in the model.

DISCUSSION

In the face of an environmental change, an adaptive physiological response can increase the chances of short-term survival (Schreck et al. 2001; Guest et al. 2016). Cortisol is a ubiquitous corticosteroid hormone that is released under acute and chronic stressful conditions in teleost fishes, and when quantified, it can provide vital information about the physiological state of an organism (Ramsay et al. 2009; Barton 2002; Mommsen et al. 1999; Wendelaar Bonga 1997). For euryhaline fishes that exist along a salinity gradient, changes in ambient salinity pose a challenge to maintenance of the internal osmolality of the fish. Cortisol plays an essential role in stimulating

the transport of ions and for proper restructuring of their gills to maintain ionic homeostasis (McCormick 2011; Tort 2011). Here, we quantified free cortisol levels in a euryhaline fish, *P. picta*, that exists along a continuum of freshwater, adjacent brackish, and saltwater habitats on the island of Trinidad. Our objective was to test whether the stability of the salinity environment was predictive of their free cortisol levels. We found that fish from environments where salinities remain stable throughout the day (freshwater (0 psu) and saltwater (20+ psu)) have significantly lower concentrations of free cortisol than populations from fluctuating brackish environments (1-19 psu) (Figure 4.2). By titrating both salt (treatment) and freshwater (control) into the aquaria of wild-caught fish in the lab, we were able to confirm that increasing salinity causes a significant spike in free cortisol concentrations (Figure 4.3). Together these results support our predictions that an acute response to changes in salinity result in elevated cortisol. Indeed, researchers have previously investigated the effects of salinity on cortisol concentrations in other euryhaline fishes in controlled lab settings (Mozanzadeh et al. 2021; Tsuzuki et al. 2007; Hegab and Hanke 1984). However, to the best of our knowledge, this is the first study to characterize cortisol levels under stable and fluctuating salinities in natural populations. We were able to obtain these results despite a long lag time between the collection of the samples and their processing by preserving fish in ethanol and then extracting cortisol at later time.

Beyond osmoregulation, cortisol has numerous other physiological roles (e.g., metabolism, growth, stress, immune function) which could be impacted under elevated cortisol levels. In most vertebrates, cortisol is the primary glucocorticoid, which binds specifically to the glucocorticoid receptor (GR), whereas aldosterone, the primary mineralocorticoid, binds specifically to the mineralocorticoid receptor (MR) (McCormick et al. 2008). However, since teleost fishes either lack aldosterone completely or contain trace amounts of the mineralocorticoid, there is support for

the concept that cortisol plays a dual role as both a glucocorticoid (to influence both metabolism and growth) and mineralocorticoid (water and ionic regulation) (McCormick et al. 2008; McCormick 2006). When a fish is subjected to a salinity stress, osmoregulatory mechanisms must be employed and energy is expended (Ern et al. 2014), wherein metabolism, and thus growth and activity, are negatively affected (Varsamos et al. 2005). Elevated levels of cortisol as a result of acute and chronic stress have also been shown to negatively affect growth while causing immune depression in tilapia (Ashley 2007). Thus, in the absence of compensatory responses, the elevated cortisol levels we observed in fluctuating salinity habitats could potentially result in a trade-off between the necessity to maintain ionic homeostasis and other traits related to fitness (e.g., swimming, growth, food acquisition, immune responses etc.). Indeed, we observed that *P. picta* experienced a decrease in escape performance 24 hours after transfer from freshwater to brackish water followed by a recovery 24 hours later (Chapter 3). Whether this change in performance reflects the direct effects of elevated cortisol or other indirect effects remains an area for future research. Nevertheless, there is a need for more studies that investigate whether euryhaline fish living in variable salinity environments face unique challenges.

This study was facilitated by the development of a new method of extracting free cortisol from fish preserved in ethanol. Our validated results from the field supported our general prediction that an acute response to a salinity challenge increases cortisol levels (Figure 3). We further validated this method by comparing cortisol levels between control and stressed fish (Figure 4). Although not previously appreciated, free cortisol appears to leach out of fish and into ethanol. Indeed, when we assayed ethanol preserved fish for cortisol, none was detected, further confirming the cortisol had leached into the ethanol. Conventional methods for measuring cortisol involve extraction from blood plasma/serum (Foster and Dunn 1974), quickly centrifuging samples to separate plasma

from blood, followed by quantification using a specific radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (EIA) (Foster and Dunn 1974; Barton et al. 1987). However, handling fish to obtain blood samples is a stressful process that could lead to an inaccurate depiction of cortisol measurements prior to handling the fish. More recently, water-borne hormone analyses have received a lot of attention since they allow for cortisol to be sampled from smaller, living aquatic organisms where blood concentrations are not sufficient for quantification (Scott and Ellis 2007). However, by extracting cortisol from ethanol-preserved fish, we can also obtain accurate whole-body concentrations using a technique that requires minimal equipment at the time of sampling, allows for long-term storage, and reduces stress on the fish. Indeed, the stability of cortisol in ethanol provides the opportunity to conduct sampling over a significant period of time, with a long lag time before the collected samples are analyzed back in the lab. In our samples, the ethanol preserved fish were stored for 2-24 months, depending on the experiment, before cortisol extractions took place. Through this new method, we provide the potential for similar studies to occur in a variety of natural populations, both terrestrial and aquatic.

Future work could expand upon this study to better understand how free cortisol levels in *P. picta* vary over time through repeated sampling of the same populations. For example, while we know salinity levels fluctuate, we do not know how frequently salinity levels drop below or exceed the isosmotic point and whether cortisol levels are constitutively elevated or track environmental salinity. In addition, future work would tease apart the variety of other factors that could be contributing to elevated cortisol levels in fluctuating environments. For example, fish in fluctuating environments may experience higher flow, causing them to have to swim against currents more than those in stable environments. The necessity for increased movement in the face of a salinity challenge could thus jointly contribute to elevated cortisol levels. For the new method,

we only validated the accuracy of quantifying cortisol in small fish that weigh less than 1 gram. Future work could investigate the efficacy of this method on larger fish to explore whether all of the cortisol leaches into the ethanol, as with *P. picta*, or if additional procedures (e.g., slicing of the fish's exterior) are necessary in order for the ethanol to penetrate fully into the fish.

Conclusion. Euryhaline fish provide a unique opportunity to understand how populations can fare in varying salinity environments. On the island of Trinidad, *P. picta* is found in both freshwater and adjacent brackish and saltwater environments, and this pattern is repeated in many streams on the island (Torres-Dowdall et al. 2013). Here, we developed a novel method of extracting free cortisol from fish preserved in ethanol and used this method to quantify cortisol levels from populations of *P. picta* that exist in natural stable and fluctuating salinity environments along a salinity gradient. After confirming that experiencing a salinity change results in increased cortisol levels in the lab, we conclude that the increased cortisol levels observed in the natural fluctuating environments conforms with predictions that cortisol plays an important role in osmoregulation. This plastic physiological response is imperative for successfully coping with salinity changes that occur as a result of daily and seasonal tidal influences in the wild.

TABLES & FIGURES

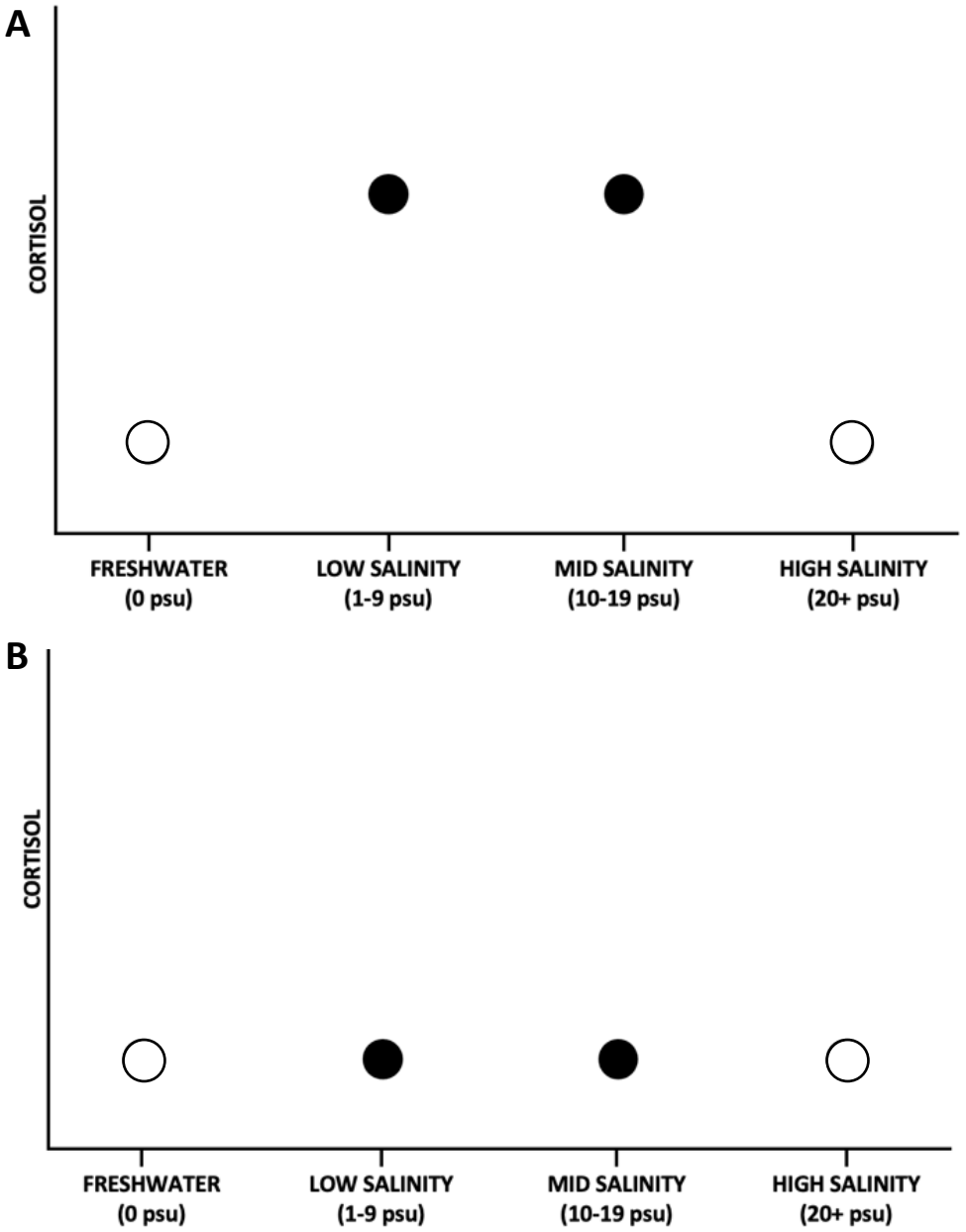

Figure 4.1. Prediction of circulating plasma cortisol levels representing **(A)** populations acclimatizing to changes in ambient salinity as a result of daily tidal fluctuations and **(B)** populations exhibiting a rapid acclimatization to fluctuating salinities in which cortisol quickly returns to baseline and thus differences are not detected. Open circles represent collection sites from ***stable*** salinity environments. Filled circles represent collection sites from ***variable*** salinity environments.

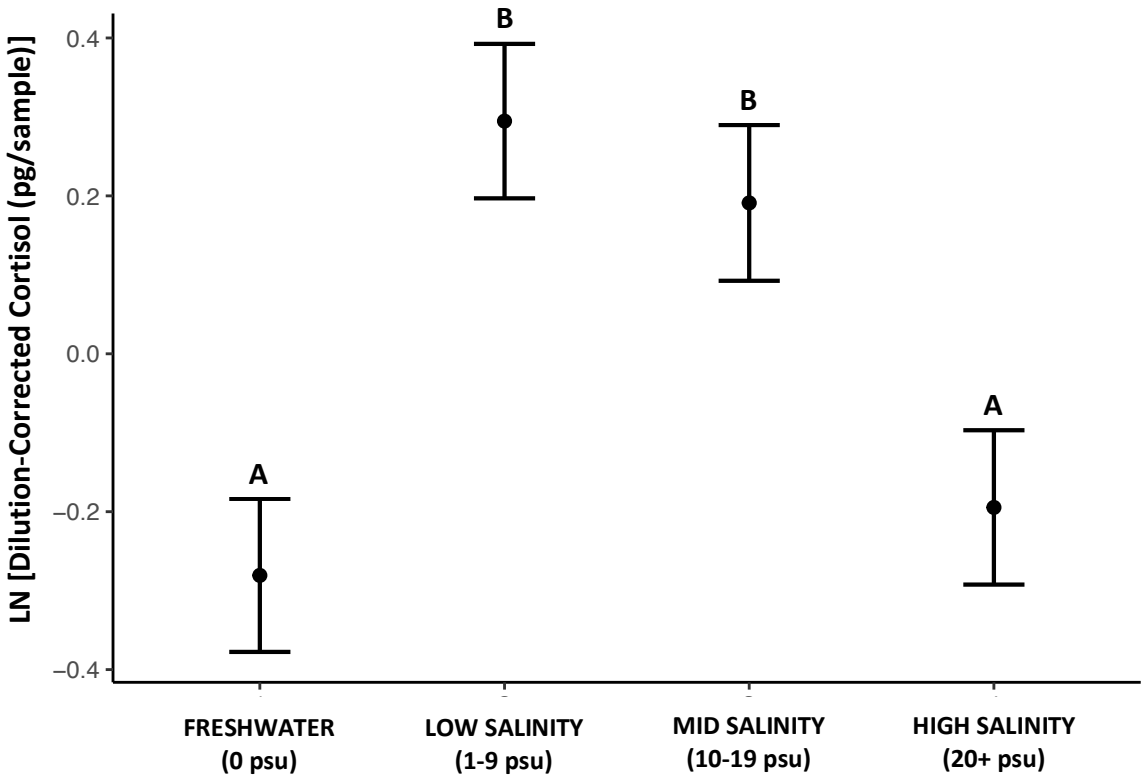

Figure 4.2. Estimated marginal means (EMMean ± SE) of the natural log cortisol x mass residuals from four different salinity levels (freshwater, low salinity, mid salinity, and high salinity) along 3 different rivers (Caroni, Caparo, and Cunupia) on the island of Trinidad. Both sex and river were included in the model.

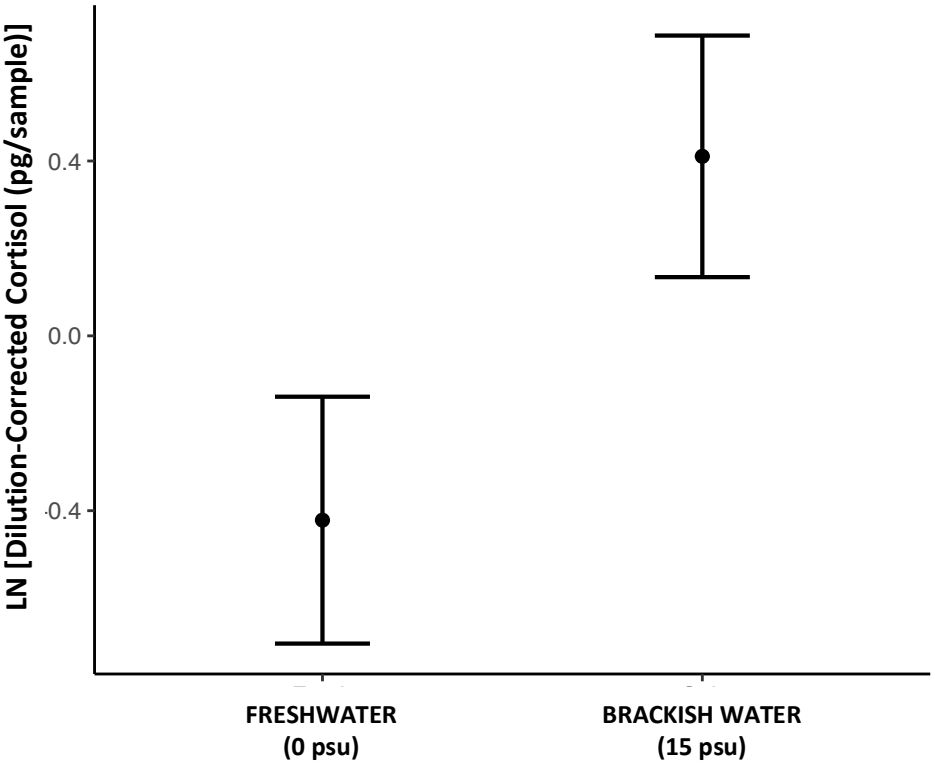

Figure 4.3. Estimated marginal means (EMMean ± 95% CI) of the natural log cortisol x mass residuals for fish titrated with fresh (0 psu) and brackish (15 psu) water. Sex was included in the model.

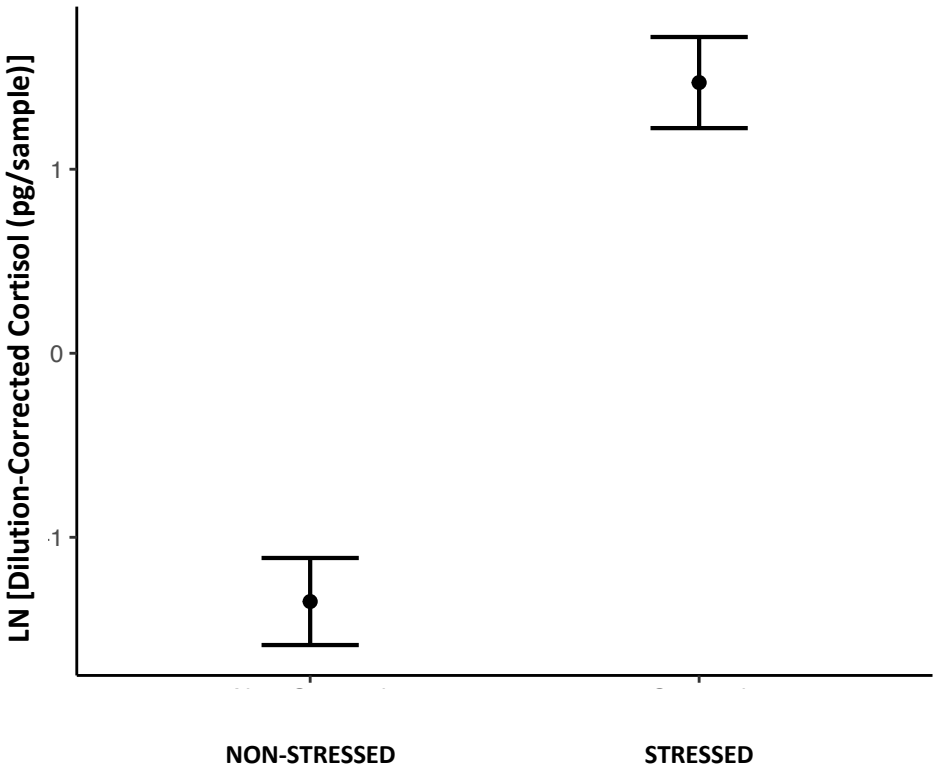

Figure 4.4. Estimated marginal means (EMMean ± 95% CI) of the natural log cortisol x mass residuals for both stressed and non-stressed fish. Results validate that the novel cortisol extraction method is effective in capturing differences in cortisol between individuals.

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APPENDIX 1: SUPPLEMENTAL TABLES & FIGURES FOR CHAPTER 2

Table 2.S1. Table depicting the acclimation and U_{CRIT} experimental schedule. For each treatment, 6 fish participated in the acclimation to ensure that at least 5 individuals survived the 24-hour acclimation period. If all 6 survived, then 5 were chosen at random to participate in the U_{CRIT} trials.

Day	U_{CRIT} Experiment	Acclimation
0	N/A	6 fish transferred to individual 0 psu containers
1	5 fish undergo U_{CRIT} trial at 0 psu	6 fish transferred to individual 0 psu containers
2	5 fish undergo U_{CRIT} trial at 0 psu	6 fish transferred to individual 6 psu containers
3	5 fish undergo U_{CRIT} trial at 6 psu	6 fish transferred to individual 6 psu containers
4	5 fish undergo U_{CRIT} trial at 6 psu	6 fish transferred to individual 9 psu containers
5	5 fish undergo U_{CRIT} trial at 9 psu	6 fish transferred to individual 9 psu containers
6	5 fish undergo U_{CRIT} trial at 9 psu	6 fish transferred to individual 12 psu containers
7	5 fish undergo U_{CRIT} trial at 12 psu	6 fish transferred to individual 12 psu containers
8	5 fish undergo U_{CRIT} trial at 12 psu	6 fish transferred to individual 15 psu containers
9	5 fish undergo U_{CRIT} trial at 15 psu	6 fish transferred to individual 15 psu containers
10	5 fish undergo U_{CRIT} trial at 15 psu	6 fish transferred to individual 18 psu containers
11	5 fish undergo U_{CRIT} trial at 18 psu	6 fish transferred to individual 18 psu containers
12	5 fish undergo U_{CRIT} trial at 18 psu	N/A

Table 2.S2. Body size measurements for length (cm), mass (g), and Fulton's body condition (g/cm^3) of fish used in U_{CRIT} trials at each of the six test salinities. Values represent the mean ± standard error.

Salinity	N	Length (cm)	Mass (g)	Body Condition (g/cm^3)
0	10	1.8045 ± 0.0359	0.055 ± 0.0048	0.0092 ± 0.0003
6	10	1.8046 ± 0.0368	0.054 ± 0.0048	0.0090 ± 0.0004
9	10	1.8272 ± 0.0287	0.056 ± 0.0027	0.0091 ± 0.0003
12	10	1.7743 ± 0.0406	0.054 ± 0.0043	0.0098 ± 0.0007
15	10	1.7787 ± 0.0305	0.053 ± 0.0040	0.0093 ± 0.0006
18	10	1.8271 ± 0.0398	0.057 ± 0.0047	0.0092 ± 0.0004

Table 2.S3. Estimated marginal mean (EMMean) values for U_{CRIT} of *P. reticulata* exposed to increasing levels of salinity.

Salinity	N	EMMean	*df*	SE	95% CI	
					Lower	Upper
0	10	20.2	53	1.83	16.5	23.9
6	10	18.2	53	1.83	14.5	21.9
9	10	20.1	53	1.83	16.4	23.7
12	10	24.5	53	1.83	20.8	28.1
15	10	17.6	53	1.83	13.9	21.3
18	10	19.3	53	1.83	15.6	23.0

Table 2.S4. Estimated marginal mean (EMMean) values for osmolality from *P. reticulata* exposed to increasing levels of salinity.

Salinity	N	EMMean	*df*	SE	95% CI	
					Lower	Upper
0	7	403	31	34.9	332	474
6	6	462	31	37.7	385	539
9	6	509	31	37.7	432	586
12	6	518	31	37.7	441	595
15	6	487	31	37.7	410	564
18	6	516	31	37.7	439	593

APPENDIX 2: SUPPLEMENTAL TABLES & FIGURES FOR CHAPTER 3

Table 3.S1. Table depicting the acclimation and escape performance experimental schedule. For each treatment, 6 fish per species participated in the acclimation to ensure that at least 5 individuals per species survived the 24-hour acclimation period. If all 6 survived for a given species, then 5 were chosen at random to participate in the escape performance trials.

Day	Escape Performance Experiment	Acclimation
0	N/A	6 fish/species transferred to 0 psu containers
1	5 fish/species undergo escape trial at 0 psu	6 fish/species transferred to 6 psu containers
2	5 fish/species undergo escape trial at 6 psu	6 fish/species transferred to 9 psu containers
3	5 fish/species undergo escape trial at 9 psu	6 fish/species transferred to 12 psu containers
4	5 fish/species undergo escape trial at 12 psu	6 fish/species transferred to 15 psu containers
5	5 fish/species undergo escape trial at 15 psu	6 fish/species transferred to 18 psu containers
6	5 fish/species undergo escape trial at 18 psu	N/A

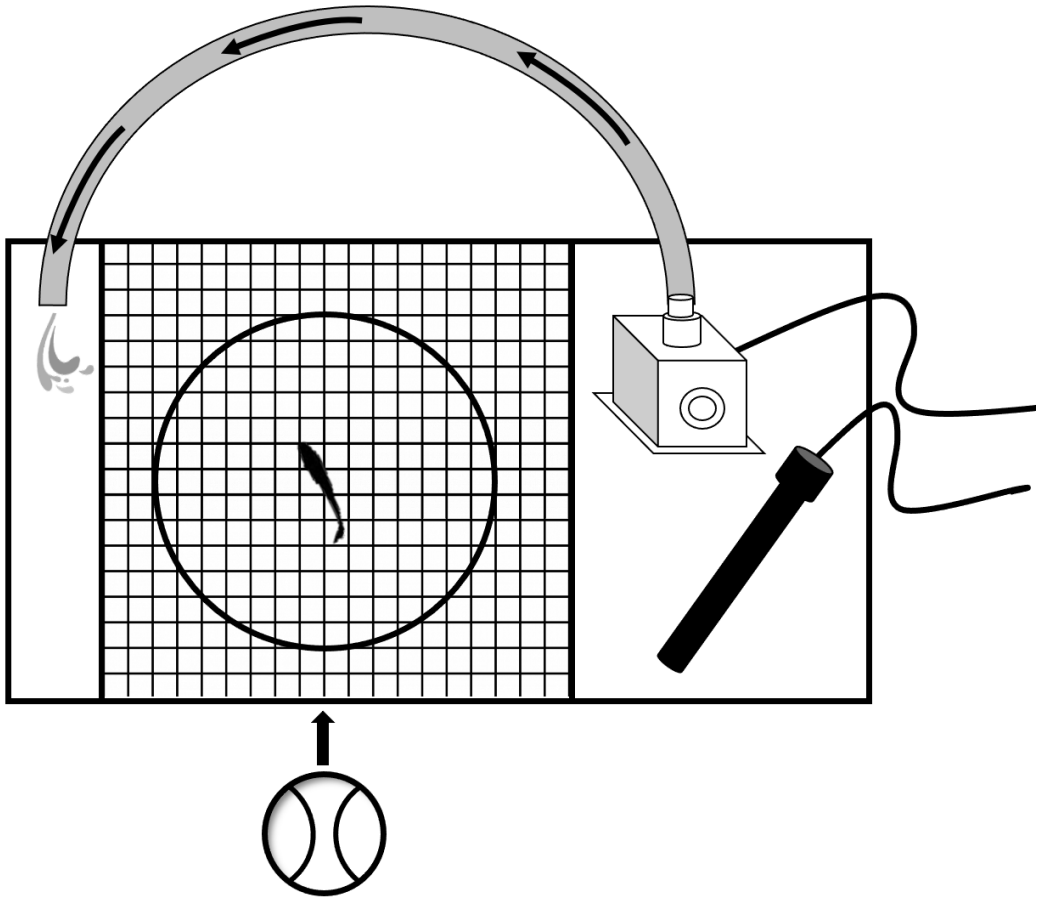

Figure 3.S1. Aerial view of experimental apparatus. The bottom of the tank was sketched with 1cm^2 boxes to determine kinematics. The right side of the tank contained a pump and heater. Water flowed from the right side of the aquarium to the left side of the aquarium via a pump and ½" tubing. Water flow allowed for oxygenation of water and heated water to flow evenly throughout the aquarium. Fish were contained within the grid-marked area via an opaque, slotted barrier to prevent view and distraction of tubing, heater, and pump, but allowed for oxygenated, heated water to infiltrate. The outside of the tank was covered in an opaque barrier to prevent distraction to the fish by the observer.

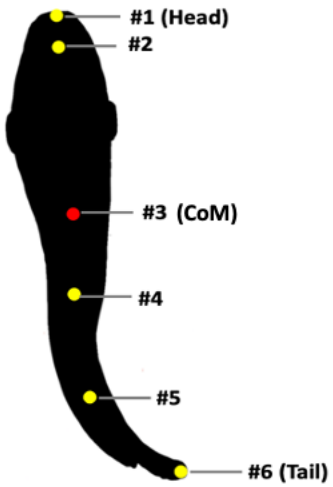

Figure 3.S2. Aerial view of 2D manual digitization/placement of 6 tracking points on fish. To determine kinematics, tracking points were placed on fish in each frame. Point #1 represents the tip of the head, point #2 represents an area between the fish's eyes, point #3 represents the center of mass (CoM), point #6 represents the tip of the tail, and points #4 and #5 were placed equidistantly between points #3 and #6.

Table 3.S2. Body size measurements for length (cm), mass (g), and Fulton's body condition (g/cm^3) of fish used in the escape performance trials at each of the six test salinities. Values represent the mean ± standard error.

Species	Salinity (psu)	N	Length (cm)	Mass (g)	Body Condition (g/cm^3)
P.reticulata	0	5	1.7758 ± 0.0372	0.0520 ± 0.0020	0.0093 ± 0.0004
	6	3	1.8713 ± 0.0785	0.0533 ± 0.0067	0.0085 ± 0.0019
	9	4	1.8010 ± 0.0123	0.0550 ± 0.0065	0.0094 ± 0.0012
	12	5	1.7842 ± 0.0262	0.0660 ± 0.0117	0.0118 ± 0.0025
	15	4	1.8528 ± 0.0516	0.0650 ± 0.0065	0.0102 ± 0.0007
	18	5	1.8090 ± 0.0843	0.0480 ± 0.0049	0.0081 ± 0.0007
P. picta	0	5	2.1438 ± 0.0560	0.1120 ± 0.0180	0.0111 ± 0.0012
	6	5	2.0670 ± 0.0520	0.0980 ± 0.0058	0.0113 ± 0.0011
	9	3	2.0957 ± 0.1426	0.0867 ± 0.0145	0.0093 ± 0.0009
	12	5	2.1252 ± 0.0527	0.0940 ± 0.0075	0.0097 ± 0.0002
	15	4	2.0465 ± 0.1013	0.0800 ± 0.0108	0.0092 ± 0.0006
	18	5	2.0604 ± 0.0607	0.0740 ± 0.0098	0.0083 ± 0.0004

Table 3.S3. Estimated marginal mean (EMMean) values for osmolality from female *P. reticulata* and female *P. picta* exposed to increasing levels of salinity.

Species	Salinity	N	EMMean	*df*	SE	95% CI	
						Lower	Upper
P. reticulata	0	3	350	28	24.1	301	399
	6	4	378	28	20.9	335	421
	9	4	370	28	20.9	328	413
	12	4	372	28	20.9	330	415
	15	4	360	28	20.9	318	403
	18	3	370	28	24.1	321	420
P. picta	0	3	421	28	24.1	371	470
	6	3	406	28	24.1	357	456
	9	2	430	28	29.5	369	490
	12	3	430	28	24.1	380	479
	15	4	406	28	20.9	363	449
	18	3	366	28	24.1	317	415

APPENDIX 3: SUPPLEMENTAL TABLES & FIGURES FOR CHAPTER 4

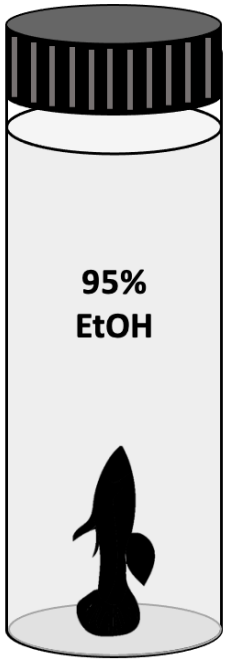

Figure 4.S1. Depiction of 40mL glass vial containing euthanized fish and 95% EtOH. The vial is about 4x the size of the female fish.

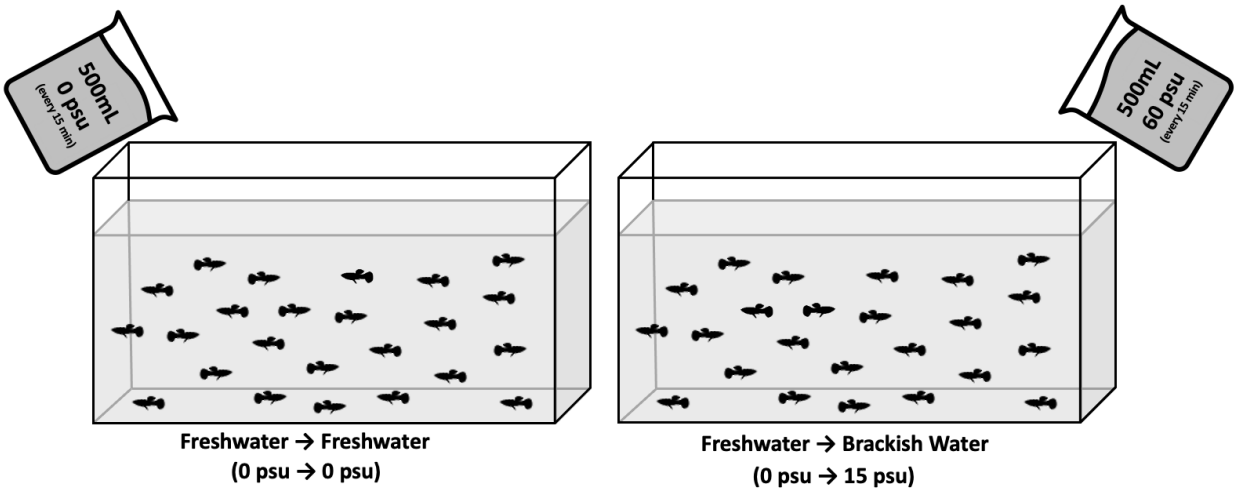

Figure 4.S2. Depiction of validation to determine effects of increasing salinity on cortisol levels. 24 individuals (12 male and 12 female) per 5-gallon aquarium were acclimated to freshwater (0 psu) for 24 hours prior to either freshwater (control group) or saltwater (experimental group) titration. Aquaria were opaque to prevent stress due to seeing the observer/titrator and fish from other aquaria.