DISSERTATION

A TAIL OF TWO FISH: AN INTEGRATIVE APPROACH TO UNDERSTAND HOW TRADE-OFFS AND SALINITY INFLUENCE TWO CLOSELY RELATED EURYHALINE FISH

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Graduate Degree Program in Ecology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2021

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ABSTRACT

A TAIL OF TWO FISH: AN INTEGRATIVE APPROACH TO UNDERSTAND HOW TRADE-OFFS AND SALINITY INFLUENCE TWO CLOSELY RELATED EURYHALINE FISH

It is well understood that adaptive evolution can occur rapidly in nature and that anthropogenic climate change is causing - and will continue to cause - mass extinctions of the planet's biodiversity. These facts represent somewhat of a paradox: rapid adaptation can and does occur in nature, yet many populations are failing to adapt to environmental change. This dissertation lies at the interface of this paradox as it investigates the adaptive process. However, instead of investigating a case of adaptive success, it explores the mechanisms and circumstances underlying a case when evolution appears to be constrained. More specifically, it investigates how a trade-off between salinity tolerance and competitive ability contributes to an evolutionary range limit in *Poecilia reticulata*. It also investigates how salinity influences genetic variation in a more widespread fish, *Poecilia picta*.

In chapter 1, a conceptual framework of trade-offs as evolutionary constraints that utilizes network/pathway thinking is presented. In chapter 2, it is experimentally shown that *P. reticulata* experiences a trade-off between salinity tolerance and competition with *P. picta*, that the trade-off is genetically based, and that it is indeed range limiting. In chapter 3 *why* this trade-off occurs at the physiological network level is investigated. It is shown that a negative relationship between salinity tolerance and competition arises because salinity exposure in *P. reticulata* results in the activation of hormonally mediated pathways in the brain associated with

ion regulation and a decrease in aggression. Chapter 4 shifts the focus from *P. reticulata* to *P. picta*. to investigate how salinity influences the distribution of both neutral and adaptive genetic variation in a species that is found both freshwater and brackish water unlike *P. reticulata*. It is found that salinity can drive differentiation at putatively adaptive loci despite high levels of population connectivity in populations of *P. picta*.

ACKNOWLEDGEMENTS

We would like to thank Karen G. Burnett, David S. Durica, Donald L. Mykles, and Jonathon Stillman for organizing and inviting us to participate in the Building Bridges from Genome to Phenome: Molecules, Methods and Models Symposium at the Society for Integrative and Comparative Biology Conference 2020 and two anonymous reviewers for their useful and thoughtful comments. We thank Kyndall Zeller, Courtney Painter, Stazi Snelling, Jovan Vincent, Amber McCardle, and Yared Belay for assistance with behavioral observations. We thank Gaston Aguilera for assistance with the Y-maze and the translocation experiments in Trinidad. We thank Lisa Angeloni and Dale Broder for assistance designing the behavior experiment. We thank Richard Evans, Porsche Robison, and Craig Marshall for assistance collecting *P. picta*. We thank Hailey Molina for assistance presenting the growth rate data. We thank Dr. Chris Funk's lab for important feedback regarding the experimental design and analyses. We thank Daryl Trumbo for assistance with DNA extractions. We thank Jennifer Roach and the other members of Dr. Andrew Whitehead's lab for assistance during library preparation and sequencing. We thank Chris Funk, Ruth Hufbauer, and Kim Hoke for their helpful comments on this manuscript. This work was supported by a grant from National Science Foundation grant (IOS-1457383) to C.K.G and by a fellowship awarded to A.A.M that is included in National Science Foundation grant (DGE-1450032). All procedures were conducted under the approval of Colorado State University Animal Care and Use Committee (protocol no. 17-7197A), and The Fisheries Division of the Ministry of Food Production, Land and Marine Affairs of Trinidad and Tobago.

DEDICATION

I feel extremely lucky and privileged to have been able to work on this project with so many wonderful people. There are truly too many people I could dedicate this dissertation too. I hope to thank all those people personally. Hence, I will dedicate this dissertation to those I cannot thank: the fish who were sacrificed during the making of this dissertation. I commit to using this research to conserve natural populations of fish and the natural world writ large to honor your sacrifice.

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A TAIL OF TWO FISH: AN INTEGRATIVE APPROACH TO UNDERSTAND HOW TRADE-OFFS AND SALINITY INFLUENCE TWO CLOSELY RELATED EURYHALINE FISH

> "Organic life beneath the shoreless waves
> Was born and nurs'd in Ocean's pearly caves;
> First, forms minute, unseen by spheric glass,
> Move on the mud, or pierce the watery mass;
> These, as successive generations bloom,
> New powers acquire, and larger limbs assume;
> Whence countless groups of vegetation spring,
> And breathing realms of fin, and feet, and wing.
> Thus the tall Oak, the giant of the wood,
> Which bears Britannia's thunders on the flood;
> The Whale, unmeasured monster of the main,
> The lordly Lion, monarch of the plain,
> The Eagle soaring in the realms of air,
> Whose eye undazzled drinks the solar glare,
> Imperious man, who rules the bestial crowd,
> Of language, reason, and reflection proud,
> With brow erect, who scorns this earthy sod,
> And styles himself the image of his God;
> Arose from rudiments of form and sense,
> An embryon point, or microscopic ens!"
>
> --- Erasmus Darwin, *The Temple of Nature*

Preface

The opening quote is a Darwin quote…but not a Charles Darwin quote. Rather, it is an excerpt from a long-form poem written by Erasmus Darwin, Charles Darwin's grandfather. I chose to include it for two reasons: 1) to continue the storied tradition of quoting "a Darwin" in a biology dissertation, & 2) to convey the simple sentiment that the concept of evolution captures the imagination. Although, Erasmus Darwin would never clearly articulate the concept of evolution like his grandson, he clearly was thinking about it. What is more, he saw enough

inherent beauty in this idea to write a poem about it. And he was not the first to feel this way. One can trace musings on what would be called evolution to many ancient cultures and a fascination with "natural history" even further back (Hull 2002). Just like humans yearned to understand why objects fell and what stars were, we yearned to understand the processes that create the biodiversity that surrounds us. When Charles Darwin proposed the Darwin/Wallace concept of evolution by natural selection (Darwin 1859), it offered an answer to that yearning and did so in way, that I would argue, captured people's imagination. Certainly, evolution and the natural world has captured my imagination. As you read this dissertation, I hope you can imagine yourself where I was during the making of it: knee-deep in the swamps of Trinidad, net in hand, trying to catch small colorful fish as the tropical sun beat down on me, my fellow scientists, and the fascinating creatures that shared the swamp with us that day and who were integral in generating the patterns of evolution that we were investigating. Evolutionary biology is a science, but, like anything worth studying, it is also much more.

Main Text

Roughly 100 years after Darwin published his seminal work, another pivotal point in evolutionary thought occurred. In the latter half of the 20th century, scientists conducted studies which demonstrated that rapid adaptative evolution can, and does, occur in nature (Losos 2017). Before this, evolution had been largely viewed as a slow, gradual process except for under very particular circumstances (Losos 2011; Losos 2017). Soon studies that examined the mechanisms and situations in which rapid adaptive evolution occurs began accumulating (Endler 1986; Reznick and Ghalambor 2001; Carroll et al. 2007) and the possibility that populations could rapidly adapt to changes in their environment became a must-consider hypothesis in evolutionary and ecological studies alike (Thompson 1998).

Around the same time of this paradigm shift in evolutionary thinking, scientists made another dramatic and consequential discovery: anthropogenic climate change was causing, and was going to continue to cause, mass extinctions of our planet's biodiversity (Malcolm et al. 2006; Botkin et al. 2007). Suddenly, somewhat of a paradox existed: rapid adaptation can and does occur in nature, yet many populations are failing to adapt to environmental change (Futuyma 2010). It became urgent for scientists to try to leverage an understanding of rapid adaptation to predict how populations would respond evolutionarily to environmental change (Bocedi et al. 2013; Valladares et al. 2014; Kellermann et al. 2020). Predicting evolution had always been one biology's "holy grails" (Losos 2017) and climate change raised the stakes exponentially.

This dissertation lies at the interface of this paradox. However, instead of investigating a case of adaptive success to better understand the adaptive process, it explores the mechanisms and circumstances underlying a case when evolution appears to be constrained. The focal species of this dissertation is the Trinidadian guppy, *Poecilia reticulata*. *P. reticulata* has historically been a prime example of how populations can rapidly adapt to new environments (Reznick et al. 1997; Magurran 2005). However, in its native range of Trinidad, their distributions exhibit an abrupt limit at the freshwater-brackish water boundary across all lowland rivers (Torres-Dowdall et al. 2013). This occurs despite studies showing Trinidadian guppies can tolerate, reproduce in, and evolve improved tolerance to saltwater in the lab (Gibson and Hirst 1955; Shikano and Fujio 1998; Shikano et al. 2001) and persist in brackish, marine, and hypersaline water in parts of their non-native range (Courtenay et al. 1974). Additionally, there are no physical or hydrological barriers to dispersal across this boundary. Hence, this range limit potentially represents an

evolutionary range limit: a range limit occurring because a population is unable to adapt to conditions beyond its range (Holt 2003; Polechová and Barton 2015).

Evolutionary range limits are excellent systems to examine the limits to the adaptive process. This is because evolutionary theory suggests that the same factors that can constrain adaptation, such as a lack of genetic variation (e.g. Blows and Hoffmann 2005), the introduction of maladaptive alleles through gene flow (e.g. gene swamping; Tigano and Friesen 2016), and the presence of genetically based trade-offs between traits (Hoffmann and Parsons 1994; Hoffmann and Blows 1994; Roff and Fairbairn 2007; Martin 2015; Mauro and Ghalambor 2020) can create evolutionary range limits (Kawecki 2008; Gaston 2009; Willi and Van Buskirk 2019). To put it more simply, the evolutionary mechanisms underlying range limits are examples of the limits of adaptation in nature, and thus range limits can be "testing grounds" to better understand the adaptive evolutionary process (Sexton et al. 2009).

The strongest experimental design to investigate the cause of an evolutionary range limit is to systematically test each potential alternative hypothesis (lack of genetic variation, gene swamping, and so on…), but that is not possible in most study systems (Willi and Van Buskirk 2019). In this dissertation, my co-authors and I instead focus on investigating what we believe is the most likely cause of the observed range limit by leveraging knowledge from the wonderful works already conducted on *P. reticulata*, Trinidad, and fish in general. For instance, Torres-Dowdall et al (2013) documented that *Poecilia picta*, a euryhaline poecilid closely related to *P. reticulata*, occurs across the salinity gradient in Trinidad and briefly overlaps with *P. reticulata* in freshwater in a pattern repeated across lowland rivers in Trinidad. Due to the nature of these two species' distribution pattern, we hypothesized that *P. reticulata* is restricted to freshwater because *P. picta* is competitively dominant under saline conditions. We predicted that this

relationship arises because osmoregulation is energetically demanding and pleiotropically linked to aggression (which contributes to competitive ability in fish), and thus the energetic demands and pleiotropic nature of osmoregulation would decrease aggression in saline conditions (e.g. Boeuf and Payan 2001; Tseng and Hwang 2008; Mauro and Ghalambor 2020). Hence, we hypothesized that *P. reticulata's* evolutionary range limit is ultimately due to a trade-off between salinity tolerance and competition. In this dissertation, my co-authors and I systematically address this hypothesis in chapters 1-3. In chapter 4 we examine how salinity influences the distribution of neutral and adaptive genetic variation in *P. picta*, which can inhabit both freshwater and brackish water, unlike *P. reticulata*.

In chapter 1, a conceptual framework of trade-offs as evolutionary constraints that utilizes network/pathway thinking is constructed to aid the experiments in chapters 2 and 3. In this chapter, my co-author and I first review the classic paradigms in which physiologists and evolutionary biologists have studied trade-offs and highlight the ways in which network and molecular pathway approaches unify these paradigms. We discuss how these approaches allow researchers to evaluate why trade-offs arise and how selection can act to overcome trait correlations and evolutionary constraints. We argue that understanding how the conserved molecular pathways are shared between different traits and functions provides a conceptual framework for evolutionary biologists, physiologists, and molecular biologists to meaningfully work together towards the goal of understanding why correlations and trade-offs occur between traits. Lastly, we highlight how the hormonal control of osmoregulation is a prime example of how a pathway approach can reveal why trade-offs can occur between seemingly unrelated traits.

In chapter 2, my co-authors and experimentally show that *P. reticulata* experience a trade-off between salinity tolerance and competition with *P. picta*, that the trade-off is

genetically based, and that it is indeed range limiting. Specifically, we use a field experiment to show the trade-off occurs in nature, a common garden breeding experiment to show the trade-off has a genetic basis, and a behavior study to show that the decline in competitive ability associated with the trade-off is likely due to *P. picta* becoming dominant over *P. reticulata* when the fish go from freshwater to brackish water. This chapter helps establish that the hypothesized trade-off exists, but it does not explicitly address *why* it occurs at the physiological network level. In chapter 1, we posited that understanding this *why* is key to evaluating trade-offs as constraints on evolution. Thus, we seek our "*why"* in chapter 3.

In chapter 3, my co-author and I examine if pleiotropy in the physiological networks underlying salinity tolerance and aggression can help explain *why P. reticulata* suffers from the range-limiting trade-off identified in chapter 2. To do this, we exposed *P. reticulata* to a salinity challenge in the presence and absence of *P. picta* and measured transcriptomic responses in the brain. We used a network analysis to uncover that there is a negative relationship between aggression and the expression of genes important to salinity tolerance. We found that this negative relationship arises because salinity exposure results in the activation of hormonally mediated pathways in the brain associated with ion regulation and a decrease in aggression. Further, we found that the specific architecture of this network indicates that adaptive amelioration of this trade-off may be difficult because of high levels of connectivity within the network. Overall, we demonstrate how overlapping biological pathways can help explain why a trade-off occurs in *P. reticulata*.

Chapter 4 shifts the focus from *P. reticulata* to *P. picta*. *P. picta* is an excellent contrast to *P. reticulata* because it is a closely related species that can span the entire salinity gradient unlike *P. reticulata*. Hence, comparisons between the two species will lead to important insights

into how different species can evolve in response to the same environmental challenge (salinity). However, before such important comparisons can be made, it is necessary to broadly understand how salinity has influenced the genetic variation present in populations of *P. picta* distributed along the salinity gradients in Trinidad. This was the goal of chapter 4. In this chapter, my co-authors and I sequenced the genomes of *P. picta* along salinity gradients in three different rivers in Trinidad. We found low levels of population structure between populations of *P. picta*, yet still found evidence for salinity's ability to drive differentiation at putatively adaptive loci. However, we did not find evidence for local adaptation in populations at the extreme ends of the salinity gradient. Ultimately, this chapter adds to the growing evidence that salinity has the power to influence genetic variation in fish over short distances in estuaries.

Overall, this dissertation demonstrates that trade-offs can be a powerful constraint to niche expansion, that network approaches can illuminate the underlying source of trade-offs, and that salinity can impact the distribution of genetic variation even over short distances. One of the consequences of climate change is that rising sea levels will increase the salinity of coastal rivers and estuaries as seawater intrudes upstream (Rice et al. 2012; Liu and Liu 2014; Renaud et al. 2015; Ghalambor et al. 2021). Hence, the results here could impact how the effects of climate change are managed in natural populations of fish. Moving forward in my career, I strive to work with management agencies so I can put this research in an applied conservation context.

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CHAPTER 1:

TRADE-OFFS, PLEIOTROPY, AND SHARED MOLECULAR PATHWAYS: A UNIFIED VIEW OF CONSTRAINTS ON ADAPTATION[1]

SUMMARY

The concept of trade-offs permeates our thinking about adaptive evolution because they are exhibited at every level of biological organization, from molecular and cellular processes to organismal and ecological functions. Trade-offs inevitably arise because different traits do not occur in isolation, but instead are imbedded within complex, integrated systems that make up whole organisms. The genetic and mechanistic underpinning of trade-offs can be found in the pleiotropic nodes that occur in the biological pathways shared between traits. Yet, often trade-offs are only understood as statistical correlations, limiting the ability to evaluate the interplay between how selection and constraint interact during adaptive evolution. Here, we first review the classic paradigms in which physiologists and evolutionary biologists have studied trade-offs and highlight the ways in which network and molecular pathway approaches unify these paradigms. We discuss how these approaches allow researchers to evaluate why trade-offs arise and how selection can act to overcome trait correlations and evolutionary constraints. We argue that understanding how the conserved molecular pathways are shared between different traits and functions provides a conceptual framework for evolutionary biologists, physiologists, and molecular biologists to meaningfully work together towards the goal of understanding why correlations and trade-offs occur between traits. We briefly highlight the melanocortin system

[1] Publishing Information: Alexander A. Mauro and Cameron K. Ghalambor

and the hormonal control of osmoregulation as two case studies where an understanding of shared molecular pathways reveals why trade-offs occur between seemingly unrelated traits. While we recognize that applying such approaches poses challenges and limitations particularly in the context of natural populations, we advocate for the view that focusing on the biological pathways responsible for trade-offs provides a unified conceptual context accessible to a broad range of integrative biologists.

INTRODUCTION

The concept of trade-offs plays a central role in biology (Stearns 1989; Kitano 2004; Roff and Fairbairn 2007; Guillaume and Otto 2012; Garland 2014; Bourg *et al.* 2019; Chen and Zhang 2020). Trade-offs are exhibited at every level of biological organization, from molecular and cellular processes (Flatt and Kawecki 2007; Campos *et al.* 2016; Sheftel *et al.* 2018) to organismal and ecological functions (Ghalambor *et al.* 2004; Simmons and Emlen 2006; Olsen *et al.* 2019). Biological trade-offs can generally be defined as the condition when a beneficial change in one trait or function results in a detrimental change to another trait or function (Stearns 1989). The presence of such trade-offs inevitably arise because different traits, functions, phenotypes, and almost all biological processes do not occur in isolation, but instead are imbedded within highly integrated and hierarchical systems and networks that make up whole organisms (Wagner and Altenberg 1996; Wagner and Zhang 2011; Hill and Zhang 2012; Murren 2012; Bourg *et al.* 2019). In this context, biological trade-offs are no different than those found in any complex system, in that multiple interacting parts must work together to carry out particular functions. Yet, such complexity and integration also leads to a fundamental dilemma often referred to as the "cost of complexity"; when many interacting parts are needed to successfully carry out a function, changing any one part will inevitably negatively impact other

traits, altering function and potentially reducing overall performance or fitness (*e.g.* Wagner and Altenberg 1996; Orr 2000a; Welch and Waxman 2003; Wainwright *et al.* 2005; Wagner *et al.* 2008). For example, consider the complexity of all the interacting elements of the mTOR signaling pathway, that receives and integrates signaling molecules from a wide range of environmental stimuli (*e.g.* nutritional status, hypoxia, insulin, stress, growth factors) and in turn interacts with numerous proteins to control many of the most fundamental cellular processes (reviewed in Kim and Guan 2019; Liu and Sabatini 2020). Such complexity represents a major challenge in attempts to understand the diverse molecular, developmental, and physiological interactions that transform genetic variation into phenotypic variation (Burnett et al. in press), or what is referred to as the *genotype-phenotype map* (*e.g.* Alberch 1991; Wagner and Altenberg 1996; Wagner 2007; Rockman 2008; Wagner and Zhang 2011).

The relationship between genetic variation and phenotypic variation is an inherently non-linear one, as a diversity of causal factors must jointly interact with each other to carry out all basic functions. The genotype-phenotype map is a non-trivial and fundamental challenge because it requires understanding how variation in individual genes and interactions across the genome (*e.g.* epistasis, pleiotropy) are propagated via molecular interactions that originate in individual cells and feed into various molecular pathways and networks to determine complex phenotypes (Soyer 2012). Yet, despite the recognition that the genotype-phenotype map requires embracing the complexity of highly integrated organismal systems, empirical approaches have largely struggled to capture the degree of interconnectedness between different levels of biological organization (*i.e.* from genes to proteins to functional phenotypes) and between different mechanisms (*e.g.* mutation, transcription, RNA editing, protein function, etc..). Instead, the study of mechanisms that transform genomic variation into phenotypic variation are dominated by

reductionist approaches that reduce the overwhelming complexity into more manageable components. For example, advances in cellular and molecular biology now allow an understanding of the independent and collective contributions of gene products (*e.g.* transcriptomes, proteomes), the regulatory control of these products (*e.g.* hormones, cytokines, and other signaling molecules), how these products interact (*e.g.* protein-protein interactions) and ultimately the pathways these products participate in and the functions they determine (Lodish *et al.* 2008). These approaches have generated large data sets and made significant progress towards understanding the set of genes, proteins, and other molecules that make up certain pathways, networks, and phenotypes (*e.g.* signal transduction, gene regulation, metabolic pathways). However, far less attention has been given to how these mechanisms and pathways interact and result in trade-offs at the level of whole organisms (Arnold 1992; Solovieff *et al.* 2013; Sommer and Mayer 2015). Here, we are interested in the mechanistic basis of trade-offs and advocate for incorporating the study of molecular pathways into evolutionary and physiological approaches to better understand the causes and constraints imposed by trade-offs. We first present an overview of the traditional and emerging conceptual frameworks used by evolutionary biologists and physiologists to study and understand the mechanistic basis for trade-offs and how these trade-offs ultimately constrain adaptive evolutionary change. We argue that these complimentary approaches find common ground when they focus on the degree to which molecular pathways and networks are shared between different functions and on the origins of these shared pathways. We then highlight some established and emerging models for studying how shared pathways result in trade-offs between different functions, and the consequences for constraining adaptive evolution.

Traditional Frameworks for Studying Pleiotropy and Trade-Offs

Evolutionary biologists have long been interested in the presence, causes, and consequences of trade-offs and recognize that many traits are not inherited independently, but rather as genetically correlated or linked traits that can trade-off against one another. In "*The Genetical Theory of Natural Selection*" Ronald Fisher was perhaps the first to explicitly relate trade-offs and organismal complexity with his geometric model of adaptive evolution (Fisher 1930). Fisher argued that organisms were like microscopes, in that they operated best when all possible adjustments (think the turning of the different knobs of a microscope) were done in harmony, such that a mutation (or adjustment of a knob) would more likely be beneficial if it had a small effect size (*i.e.* a small adjustment as opposed to large adjustment of the knob) because it would be less likely to have a large impact on multiple traits and compromise function. Implicit in Fisher's model was the concept of *pleiotropy*: the ability for a single gene to affect more than one trait (see Figure 1 for a simple example of genetic pleiotropy where a single gene affects two traits; Hodgkin 1998; Paaby and Rockman 2013). Pleiotropy describes the pattern by which different traits and functions are genetically connected or correlated, and how pleiotropy manifests itself in a system determines the pattern of trade-offs and constraints. For example, under the idea of "universal pleiotropy" any mutation at any locus has the potential to affect every trait either directly or indirectly. Under the assumption of universal pleiotropy, the cost of complexity is high as trade-offs between traits would be very common. Conversely, if pleiotropy is rare, then the cost to complexity is alleviated because traits are free to change without impacting other traits or functions (Stearns 2010; Paaby and Rockman 2013).

In the context of trade-offs, the primary focus of evolutionary biologists has been on *antagonistic pleiotropy*, or when a single gene affects two traits in opposing directions, such that increasing one trait value results in a decrease in the value of the other trait (with increased trait

values being associated with fitness; Paaby and Rockman 2013). For example, Figure 1 illustrates a scenario where a pleiotropic gene codes for two distinct molecular functions that result in an increase in trait 1 that increases fitness and a decrease in trait 2 which decreases fitness: antagonistic pleiotropy. The presence of antagonistic pleiotropy has far reaching implications because it provides an explanation for why natural selection is unable to simultaneously optimize multiple traits and how improvement in one function can cause reduced fitness through correlated changes in other functions. Testing for the presence of antagonistic pleiotropy has historically relied on a correlative and statistical approach based on breeding and artificial selection experiments to estimate the degree to which different traits are genetically correlated with each other (Stearns 2010). Such approaches have found unexpected genetic correlations among traits. For example, selective breeding for high voluntary wheel-running behavior in mice has led to a suite of correlated evolutionary changes in almost every component of the phenotype from morphology (*e.g.* Castro and Garland 2018), physiology (*e.g.* Hiramatsu and Garland 2018), to the neuro-endocrine system (Garland *et al.* 2016). Further, recent studies have combined artificial selection experiments with genome wide association studies (GWAS) to uncover candidate pleotropic genes that could underlie observed trait correlations and trade-offs (*e.g.* Dong *et al.* 2019). Collectively, such results highlight not only the magnitude of interconnectedness and integration at the whole organism level, but also the potential constraints imposed on traits under selection to independently evolve (Garland *et al.* 2016). The recognition of such genetically based trade-offs have been extremely influential in the design of selective breeding experiments for agricultural products (Falconer and Mackay 1996; Chen and Lübberstedt 2010), the evolutionary theories for senescence (*e.g.* Williams 1957), life history

evolution (*e.g.* Roff 1992; Stearns 1992), adaptation (*e.g.* Bennett and Lenski 2007), and the maintenance of genetic variation (Falconer and Mackay 1996).

Physiologists have also had a long history of interest in trade-offs at multiple levels of biological organization. All physiological and biochemical processes are ultimately grounded in the constraints imposed by physical and chemical laws, which in term place limits on the range of available adaptive solutions to environmental challenges (*e.g.* Garland and Losos 1994; Somero et al. 2017). However, physiologists and evolutionary biologists have increasingly found common ground by investigating the mechanisms underlying how finite resources are allocated to the competing demands of growth, developmental, reproduction, and maintenance (*e.g.* Sibly and Calow 1986; Zera and Harshman 2001; Ricklefs and Wikelski 2002; Flatt and Heyland 2011). Such allocation trade-offs commonly underlie life history and developmental trade-offs. For example, Zera *et al.* (1998) experimentally demonstrated the role of juvenile hormone (JH) in controlling the allocation of nutrients between ovaries and flight muscle; an allocation trade-off that underlies two distinct life history strategies associated with early reproduction versus flight capability. Similarly, JH also plays a role in the allocation of resources to different body parts during development, such as between the size of beetle horns and eyes (Nijhout and Emlen 1998). The multiple roles of JH exemplify a general role played by hormones in generating trait correlations and trade-offs (Ketterson and Nolan 1999; Zera *et al.* 2007; McGlothlin and Ketterson 2008; Ketterson *et al.* 2009; Hau and Wingfield 2011; Cox *et al.* 2016; Dantzer and Swanson 2017). How hormones act pleiotropically on diverse suites of traits encompassing morphology, physiology, and behavior, and how changes in hormone receptors can alter tissue sensitivity to break up these pleiotropic effects are major themes in the emerging field of evolutionary endocrinology (Zera *et al.* 2007; McGlothlin and Ketterson 2008; Ketterson *et al.*

2009; Hau and Wingfield 2011; Cox *et al.* 2016; Dantzer and Swanson 2017). An example of hormonal pleiotropy is also depicted in Figure 1 where one hormone interacts with receptors that underly the two different traits. Now, one can argue that this is still just an example of genetic pleiotropy as the hormone originates from a single gene. However, by understanding the pathway responsible for the trade-off, one gains a more precise understanding of the roles played by different gene products and where in the pathway the trade-off originates. The development of evolutionary endocrinology as a field of study reveals that physiologists and evolutionary biologists are converging on a shared and more integrative understanding of the mechanisms that underlie trade-offs.

Trade-Offs and Pleiotropy in the Context of Network Theory

Recent developments in next generation sequencing, mass spectrometry, and other technologies have generated vast amounts of high dimensional data at different levels of biological organization (genomes, transcriptomes, proteomes, etc..), which in turn has led to new perspectives and interest in the role of pleiotropy and trade-offs. In particular, there has been a shift towards embracing perspectives based on complex systems and network theory to study patterns within large data sets, as in evolutionary systems biology (Soyer 2012; Soyer and O'Malley 2013; Melo *et al.* 2016) or in the context of physiological response networks (Cohen *et al.* 2012; Martin and Cohen 2015). These network approaches attempt to mathematically model the complex pattern of interactions underling the relationship between genomes and phenomes (Soyer 2012; Soyer and O'Malley 2013; Melo *et al.* 2016). Systems biology tools are used to dissect these properties so researchers can understand network dynamics and make informed predictions about how complex systems will respond to mutations or other perturbations (Ciaccio *et al.* 2014; Soyer 2012; Soyer and O'Malley 2013).

The topological structure of networks share common features such as individual nodes (often specific molecules or genes) that are connected to each other by edges that represent physical interactions or genetic processes, and emergent features such as modularity (*e.g.* functionally similar nodes that strongly interact with each other) and robustness (*i.e.* the ability of the network to maintain function in response to genetic or environmental perturbations; Soyer and O'Malley 2013; Ciaccio *et al.* 2014; Melo *et al.* 2016). These basic features of networks can be seen in Figure 1: signaling molecules, receptors, and genes represent nodes that are connected to one another via physical interaction (signaling molecule to receptor) or genetic processes (genes to receptors/molecules). In the context of trade-offs and evolutionary constraints, modularity is a key concept because it describes the degree to which traits are constrained or free to evolve within complex networks. Specifically, a system is modular when it "can be divided into multiple sets of strongly interacting parts that are relatively autonomous with respect to each other" (Melo *et al.* 2016). In other words, traits within a module (*e.g.* levels of gene expression, parts of a morphological trait) are highly correlated and thus constrained from independently changing due to pleiotropic interactions and shared underlying pathways, whereas separate modules are more independent of each other and free to change without compromising particular functions. Properties of networks/modules, the degree of connectivity between modules, and the way in which crosstalk occurs between modules, influences how organisms respond to environmental change and influences how modules respond to selection (Martin *et al.* 2011; Melo *et al.* 2016). Indeed, the presence of modularity is thought to be one solution to the cost of complexity (see above) because it allows for complex systems to be compartmentalized into subnetworks that minimize negative pleiotropic effects (Wagner and Altenberg 1996; Wagner 2007; Melo *et al.* 2016; Dantzer and Swanson 2017).

But how common is pleiotropy throughout the genome and what is the evidence that pleiotropy is more common within than between modules? How much does pleiotropy result in trade-offs that constrain adaptive evolution and does modularity actually lessen the constraint? New genomic tools and network analyses are providing empirical measurements of the patterns of pleiotropy across genomes. Wagner & Zhang (2011) summarized genomic studies on yeast, nematodes, mice, and humans and found that pleiotropic effects were largely modular---meaning that most pleiotropy was found within the same functional gene networks. The finding that specific biological functions have integrated genetic underpinnings was not new (*e.g.* Chesler *et al.* 2005; Wagner *et al.* 2007) but finding that pleiotropy was largely confined to integrated networks suggests that pleiotropically based trade-offs should be limited to similar biological functions (Wagner and Zhang 2011). A surprising conclusion was that pleiotropy seemed to be low across the genome (the median mutation in these studies only affected 2-8% of the traits examined; Wagner and Zhang 2011). Wagner & Zhang (2011) also found that the per-trait effect size of a mutation increased with the number of traits affected by the mutation. In their model this meant that "moderately" complex organisms had faster adaptation rates than the simplest of organisms, counter to the "cost of complexity" paradigm. Wagner and Zhang (2011) suggest that trade-offs and evolutionary constraints due to pleiotropy may not be as prominent as once thought. However, Hill & Zhang (2012) warned against any broad conclusions because the statistical framework used by Wagner and Zhang (2011) may have underestimated pleiotropy. Further, the trait changes analyzed by Wagner and Zhang (2011) were not necessarily linked to a fitness change. Traits closely linked to fitness may have a different prevalence than other forms of pleiotropy because of the complex effects of trait-trait interactions on fitness (Paaby and Rockman 2013). The directionality of this difference will be challenging to test because the

fitness consequences of trait changes are inherently difficult to measure and highly dependent on environmental context (Eguchi *et al.* 2019). Nevertheless, the degree and form of pleiotropy across the genome remains an open but critical question not only in the context of evolvability, but also in our ability to associate genetic variants to specific phenotypes (Boyle *et al.* 2017).

Physiologists have also started to incorporate network thinking by viewing physiological systems as complex networks. Viewing physiological responses as a network instead of a series of individual responses leads to explicitly considering how different physiological systems interact with each other and enables the ability to anticipate potential trade-offs. Cohen *et al.* (2012) defined Physiological Response Networks (PRNs) as "the network of molecules and their regulatory relationships that maintain and adjust homeostasis and facilitate performance at the whole-organism level." Like other types of networks, PRNs have nodes and edges, where the nodes are typically the circulating concentrations of signaling molecules (*e.g.* hormones, proteins, mRNA, etc…) and their associated receptors, whereas the edges define the co-regulatory patterns of the nodes – such as the magnitude and direction of change in one node after another node has been altered (Martin and Cohen 2015). What is appealing about PRNs is that they shift the scale of biological organization to the whole organism and identify the mechanistic interactions between whole physiological systems (Cohen *et al.* 2012; Martin and Cohen 2015). In this context, PRNs view physiological systems as being dynamic, (*i.e.* having a PRN state), such that PRN states will shift in order to achieve homeostasis or carry out a particular function (Cohen *et al.* 2012; Martin and Cohen 2015). Despite the appeal of PRNs as providing a more holistic view of how physiological states respond to environmental variation through plasticity and evolutionary change, the specific architecture of most PRNs remains largely unknown (Martin and Cohen 2015). Nevertheless, a PRN approach is particularly useful

in stimulating more critical thinking about the exact mechanisms that generate trade-offs, and how those trade-offs might be resolved. For example, both Flatt & Heyland (2011) and Hughes & Leips (2018) note that not all resource allocation trade-offs are necessarily determined by energy budgets but could alternatively be explained by the way signaling pathways are shared between different life history traits. Thus, understanding PRN architecture can help compare alternative hypotheses for why trade-offs arise between different physiological systems.

By viewing physiologically based trade-offs in the context of modularity and PRNs, a link can be made between how physiologically based trade-offs constrain or facilitate adaptive evolution. The relation to evolutionary thinking is two-sided: the rate of evolution could be slowed and the potential direction of evolution within trait space could be limited by trait correlations created by networks, or alternatively, tight trait correlations that are products of past selection could facilitate rapid adaptive change (Ketterson *et al.* 2009). For example, Aubin-Horth and colleagues found that boldness and aggression were correlated in sticklebacks because the traits share an underlying biological network (Aubin-Horth *et al.* 2012). If an environmental change jointly selects for increased boldness and aggression, then perhaps adaptive evolution would be facilitated due to the shared network. But if an environmental change requires breaking these two traits apart, adaptive evolution could be constrained. This type of network thinking can help identify spots in a physiological network where alterations would be most effective in ameliorating trade-offs. Indeed, Di Poi *et al.* (2016) studied four PRNs in freshwater and marine stickleback populations and found that adaptation to freshwater was facilitated by changing patterns of expression in specific receptors rather than global changes to the entire PRN, providing empirical evidence for the idea that modularity in biological pathways can ameliorate trade-offs due to pleiotropy. Similarly, Sommer & Mayer (2015) reviewed developmental

mechanisms in different nematodes and found a conserved "developmental switch" controlling a hormone receptor. The switch is used in many ecological functions and was the evolutionary target for the development of novel regulatory loops that altered intraspecific competition (Sommer and Mayer 2015). This switch is an example of an "integrator" (Cohen *et al.* 2012), a part of a network that connects modules and is disproportionally important in shaping trade-offs (Martin *et al.* 2011) and thus is often implicated as being key to creating or ameliorating trade-offs.

Studying Pleiotropy and Trade-Offs in the Context of Molecular Pathways

The conceptual frameworks used by evolutionary biologists and physiologists to study pleiotropy and trade-offs share one unifying component: the biological pathways that underlie the traits of interest determine the degree to which the traits are correlated. The mechanisms by which pleiotropy and trade-offs are manifested within organisms can be understood by focusing on the molecular pathways that are involved in the transmission of signals, the regulation of gene expression, and/or metabolism and how they all interact as part of a larger network (Soyer 2012). Yet, how pleiotropy manifests itself at the molecular level is complex (Figure 1), as single genes may have different molecular functions depending on context or have a single molecular function that impacts multiple biological functions (Dudley *et al.* 2005; He and Zhang 2006; Paaby and Rockman 2013; Stoney *et al.* 2015). Indeed, when one considers how the molecular function of genes change as one examines processes at the levels of single cells, tissues, or organs, and how these changes impact the physiological state of whole organisms, the definition of pleiotropy, traits, and trade-offs changes at different levels of biological organization (Paaby and Rockman 2013; Stoney *et al.* 2015). These molecular mechanisms are also dynamic and often activated and regulated by hormones in response to changing internal and external environmental conditions (*e.g.* Aranda and Pascual 2001; Cheung and Kraus 2010).

Nevertheless, by focusing on the molecular pathways influencing the traits of interest, genetic and hormonal pleiotropy converge upon studying the same set of mechanisms for the causes and consequences of trade-offs.

Evolutionary biologists and physiologists embracing molecular pathways have started to gain new insights into the causal mechanisms underlying correlations, trade-offs, and constraints in a diversity of phenotypic traits closely tied to fitness (*e.g.* Flatt *et al.* 2005; Ayroles *et al.* 2009; Chen and Lübberstedt 2010; Hau and Wingfield 2011; Schwartz and Bronikowski 2011; Schwartz and Bronikowski 2013; Aubin-Horth 2016; Saltz *et al.* 2017; Durmaz *et al.* 2019). Such integrative approaches have been dependent on advances made in cell and molecular biology and are increasingly available to a broader range of researchers because genes, gene functions, and biological pathways are largely conserved across multicellular organisms. While much work remains in understanding the complexities of gene function and molecular pathways, integrative biologists working with non-model organisms can use widely available databases to annotate gene functions (*e.g.* gene ontology "GO", see Ashburner *et al.* 2000) and tools for pathway enrichment analysis (*e.g.* KEGG, MetaCyc, Reactome, see Bindea et al. 2009; Kelder et al. 2012; Altman et al. 2013; Mykles et al. 2016; and for recomdendations for future studies see Burnett et al. in press). The challenge in most cases will be to connect the action of molecules and the associated biological pathways with ecologically relevant fitness related traits (Loewe 2012). But when these challenges are overcome, such approaches can lead to novel discoveries. For example, Ihle and colleagues (2015) investigated the mechanistic control of a complex behavioral syndrome in honeybees. Previous work had shown that two mutually suppressive hormones, juvenile hormone (JH) and vitellogenin (Vg), were largely responsible for mediating the pollen hoarding syndrome (PHS), a syndrome in which foraging, ovary size, and life history

traits are all linked. Ihle *et al.* (2015) selected for strains that differed in their strength of the PHS, then functionally reduced Vg via RNA interference in a backcross of different strains and conducted a QTL study on various phenotypes of the PHS. This methodology lead to the discovery of the genetic basis for endocrine controlled traits. Using a different approach, Ayroles *et al.* (2009) used transcriptome sequencing in 40 different inbred fruit fly lines to understand the genetic underpinnings of, among other things, 6 correlated phenotypic traits. They found statistically significant trade-offs between many of the traits (*e.g.* competition & starvation resistance and longevity & competition). They used modularity clustering techniques (Stone and Ayroles 2009) to break the traits into modules of transcripts and then looked for genes that overlapped between modules (at a rate higher than expected by chance). The researchers discovered "substantial modular pleiotropy" and used GO terms to better understand the functionality of the pleiotropic genes. For example, they found that genes affecting mitochondrial ribosomes to be pleiotropic for chill coma recovery and starvation resistance; a pattern that was otherwise not obvious. In other cases, knowledge of existing molecular pathways can be applied to better understand particular systems. Schwartz and Bronikowski (2013) provide a model approach for studying how molecular stress pathways shape life history evolution and the specific nodes within this pathway that are under selection. Similarly, Reagan *et al.* (2020) describe how the insulin like signaling pathway and related pathways such as mTOR, integrate a range of environmental inputs and provide a robust framework for understanding the relationship between dietary restriction and aging. Collectively, these approaches provide evidence for the power of focusing on the molecular pathways that connect gene functions to phenotypes, as opposed to focusing only on statistical association between specific candidate genes and phenotypes (*e.g.* GWAS studies; see also Boyle *et al.* 2017).

Inspired by this work, below we briefly highlight the melanocortin system and the control of osmoregulation, as different ways in which molecular pathways can be used to study trade-offs and make *a priori* predictions about evolutionary constraints in a genome to phenome context.

The Melanocortin System: Trade-Offs Between Coloration and Behavior

Variation in color and its correlation with other traits has been important in ecological and evolutionary studies as color-related traits are known to respond to natural selection in the wild (Hoekstra *et al.* 2004; Hoekstra *et al.* 2005), artificial selection in the lab (Rajpurohit *et al.* 2016), and have even been implicated as a potential mechanism for speciation (McKinnon and Pierotti 2010). The melanocortin system is of particular interest because it is highly conserved across vertebrates, affects many traits in addition to color (*e.g.* aggressiveness, sexual behavior, immune function, the stress response, energy homeostasis, and social behavior; see Ducrest *et al.* 2008), and does so across many different tissue types (Cone 2005; Ducrest *et al.* 2008; Roulin *et al.* 2011). Additionally, the genetic basis of the melanocortin system is relatively simple as evident by small mutations that dramatically alter coloration (Hoekstra *et al.* 2006) and can be manipulated in the lab (Matsuoka and Monteiro 2018). Overall, it is an ideal system to study trade-offs and the pathways that underly them.

The melanocortin system refers to a set of hormonal, neuropeptidergic, and paracrine signaling pathways that are defined by various components including the five G protein-coupled melanocortin receptors (*e.g.* MC1-5R); peptide agonists derived from the proopiomelanocortin preprohormone precursor (*e.g.* MSH isoforms and ACTH) which is coded for by the POMC gene; and the endogenous antagonists, agouti signaling protein (ASIP) and agouti-related protein (AGRP)(reviewed in Cone 2006). POMC is largely expressed in the pituitary gland, so melanocortins are dispersed into the bloodstream, brain, and peripheral tissues (like skin)

(Dijkstra *et al.* 2017), which means that different receptors can be expressed simultaneously across different tissues. Hence, in the absence of downstream regulation or mutations, the expression of the receptors and their corresponding effects on phenotypes should be correlated. For example, in fish alpha-MSH leads to the expression of MC1R in the skin, which leads to darker coloration, but it can also lead to the expression of MC3R & MC4R in the brain which can lead to increases in aggression by modulating the dopamine system (Dijkstra *et al.* 2017).

Because of the highly interconnected nature of the traits within the melanocortin system, and because the melanocortin system is highly conserved across vertebrates, *a priori* predictions can be made across a wide range of taxa for which traits will be correlated with color differences. Ducrest *et al.* (2008) did this in a meta-analysis and found darker colored individuals were often more aggressive and had a stronger stress response relative to their lighter colored conspecifics (Ducrest *et al.* 2008). Importantly, they were also were able to make insights as to under what conditions trait correlations between coloration and melanocortin-based phenotypes should or should not exist. Specifically, Ducrest *et al.* (2008) suggest that trait correlations should only exist when agonists causing the expression of MC1R in the skin (the cause of darker coloration) are coordinated with the expression of the other MCR subtypes across different tissues. Hence, differences in tissue specific expression of inverse agonists or mutations in MC1R can lead to individuals that only differ in coloration and not any of the potentially correlated traits. For example, in the beach mouse system where color variation between populations allow them to adaptively match the substrate they live on, the differences in color is based on a single mutation in the MC1R gene (Hoekstra *et al.* 2007) and thus the color polymorphism would not be predicted to correlate with other traits. However, when more of the melanocortin module is involved in trait expression and genetic backgrounds differ, the picture becomes more

complicated. For example, Dijkstra and colleagues (2017) studied the role of the melanocortin system in regulating color and aggression in two color morphs of the cichlid, *Astatotilapia burtoni*. They found the same phenotypic correlation between darker color and aggression in both morphs, but they also discovered that the melanocortin system is differentially activated in the two morphs after pharmacological manipulation of alpha-MSH and ASIP (Dijkstra *et al.* 2017). This suggests that breaking apart the aggression/color trait correlation would need to take different routes in the two morphs which means the corresponding evolutionary constraint could be morph specific. Similarly, morph specific regulation of the melanocortin system in the Tawny Owl*, Strix aluco,* has also been found (Roulin *et al.* 2011; Emaresi *et al.* 2013; Emaresi *et al.* 2014). In this system, darker male owls have higher survival than their lighter conspecifics and, as predicted by life history theory, produce lower numbers of higher quality offspring than lighter males (Emaresi *et al.* 2014). Interestingly, investment in offspring is relatively inflexible to stress in dark males but is flexible to stress in light males, revealing a phenotypic correlation between color, life-history, and the stress response. The authors postulated that this could be due to differences in POMC regulation because previous work showed that lighter female tawny owls had altered regulation of POMC to stress manipulation whereas their darker counterparts did not (Roulin *et al.* 2011). However, they have also found sex specific regulation of melanocortin which could further complicate the matter (Emaresi *et al.* 2013). These examples of the melanocortin system reveal how pathways are shared between traits like color, the immune system, and life history leading to morph and sex specific differences. Collectively, a deeper understanding of the melanocortin system and its main components has helped increase our understanding of why seemingly unrelated traits are correlated with each other. This provides greater power to predict *a priori* when trait correlations should occur and when they might be

broken which is imperative when assessing the degree to which trade-offs/trait correlations act as evolutionary constraints.

A Trade-Off Between Salinity Tolerance and Aggression

Osmoregulation, the process in which organisms maintain internal ion balance, is vital to aquatic organisms that experience different salinities and has been particularly well studied in fish (Marshall and Grosell 2006.; Evans, Claiborne, and Currie 2013). The physiological mechanisms underlying osmoregulation in euryhaline fish have long been recognized to be under hormonal control (reviewed in McCormick 2001; McCormick and Bradshaw 2006; Sakamoto and McCormick 2006; Mancera and Mccormick 2007). Yet, the same endocrine control mechanisms are also known to have pleiotropic effects on numerous other traits (*e.g.* Mommsen *et al.* 1999). For example, arginine vasotocin (AVT), growth hormone (GH), and cortisol (McCormick 2001; Warne *et al.* 2002; McCormick and Bradshaw 2006; Sakamoto and McCormick 2006; Mancera and Mccormick 2007) are also known to influence behavioral aggression (Jönsson and Björnsson 2002; Santangelo and Bass 2006) and metabolism (Sangiao-Alvarellos et al. 2004), leading to potential trade-offs between osmoregulation and other traits (Gilmour *et al.* 2005; Alcaraz *et al.* 2008).

How might such trade-offs arise via biological pathways and how might they manifest themselves in an ecological setting? We recently began investigating if a trade-off between salinity tolerance and competitive ability in *Poecilia reticulata*, the Trinidadian Guppy, could provide an explanation for why this euryhaline fish it is restricted to freshwater on the island of Trinidad. The guppy is native to the Caribbean, Central America and South America (Magurran 2005), and has been experimentally shown to tolerate, reproduce in, and evolve improved tolerance to saltwater (Gibson and Hirst 1955; Shikano and Fujio 1998; Shikano *et al.* 2001).

Further, it can be found in brackish waters in introduced parts of its range (Courtenay *et al.* 1974). Yet, the guppy is only found in freshwater in the streams of Trinidad despite its documented physiological tolerance to brackish and saltwater. Sampling of the abiotic and biotic factors on Trinidad show that the guppy's range limit is best correlated with a change in salinity and the presence of another closely related Poecilid, *Poecilia picta* or the Swamp Guppy (Torres-Dowdall *et al.* 2013). Hence, the movement of guppies into brackish water in Trinidad would mean increased interactions with a potential competitor in addition to dealing with increased osmoregulatory demands (see Figure 2 for an illustration of how the change from freshwater to brackish water results in a cascade of physiological responses).

The guppy's natural history and distribution suggest that the species may experience a trade-off between aggression (a proxy for competitive ability) and osmoregulation (Torres-Dowdall *et al.* 2013). But why should osmoregulation and aggression be correlated traits that trade-off against one another? We hypothesize that the answer to this question for guppies and other euryhaline teleosts is due to the overlap between osmoregulatory pathways and aggression pathways. To our knowledge, no study to date has thoroughly examined how the two biological pathways controlling osmoregulation and aggression overlap to create this trade-off, although these pathways have been well studied separately. In Figure 2 we depict a simplified model of how this trade-off may manifest via the overlap of pathways involving the pleiotropic effects of several hormones. Briefly, an increase in environmental salinity is known to cause an increase of AVT in the brain (McCormick 2001; Evans *et al.* 2005; Evans and Somero 2008; Filby *et al.* 2010; Martos-Sitcha *et al.* 2019). AVT synthesis and secretion result in the release of adrenocorticotropic hormone (ACTH) which controls the secretion of cortisol, which along with growth hormone (GH) and other hormones, increase ion transport via activation of the NKCC

ion transporter and other transporters in the gills (AVT affects the ion transporters directly too) (Mancera and Mccormick 2007; Morando et al. 2009; Lema et al. 2019; Martos-Sitcha et al. 2019). However, cortisol also acts to decrease aggression (DiBattista et al. 2005) which creates a potential for a trade-off between salinity tolerance and aggression. Interestingly, GH has been shown to increase aggression in fish (Jönsson and Björnsson 2002; Trainor and Hofmann 2006), thus it is able to increase both aggression and salinity tolerance and could perhaps ameliorate the magnitude of this trade-off. However, there is limit to how much GH can alter the trade-off. The initial increase in AVT upon entering salinity also leads to activation of the serotonin and dopamine pathways. This is beneficial in terms of ion transport as the neurotransmitters 5-HT (part of the serotonin pathway) and DA (part of dopamine pathway) regulate the release of GH. But, this has a simultaneous negative effect in terms of aggression, as 5-HT activates 5-HT receptors (HTR) and DA activates DA receptors (D1-D5) that reduce aggression (Sangiao-Alvarellos et al. 2004; DiBattista et al. 2005; Gilmour et al. 2005; Filby et al. 2010; Jeffrey et al. 2014). Hence, AVT through the intermediates of cortisol, GH, 5-HT, and DA appears to act as an antagonistic pleiotropic node. There are more candidate pleiotropic nodes that could lead to the same trade-off, but preliminary evidence from experiments that measure changes in gene expression in the brain and gills most strongly implicates the pathway described above (Mauro & Ghalambor *in prep*.). The same preliminary experiments also reveal that exposure to salinity challenge results in reduced aggression at the phenotypic level (Mauro & Ghalambor *in prep.*). By combining transcriptomics, with experimental manipulations of salinity, and the competitive environment, we are attempting to link differential gene expression and shared pathways to the phenotypes that cause a trade-off between aggression and salinity tolerance. Although many of the details on the pathways underlying this trade-off require further investigation, we believe this

approach can yield insights as to the degree this trade-off ultimately represents an evolutionary constraint.

DISCUSSION AND CONCLUSIONS

There is an increasing recognition that genetically based trade-offs, like those that arise because of pleiotropy, ultimately manifest themselves through complex biological pathways to shape whole organism physiology and fitness (Bourg *et al.* 2019). As a result of this and by examining how genomes map to phenomes, evolutionary biologists and physiologists are converging on a shared understanding of the molecular mechanisms underlying trade-offs and the constraints on the range of possible phenotypes. This is in contrast to the historical conceptual divide between how evolutionary biologists and physiologists have typically studied trade-offs. Evolutionary biologists have often treated the molecular mechanisms underlying trade-offs like a "black box," the details of which were not critical to the testing and development of theory (see discussion in Flatt and Heyland 2011 with S. Stearns). On the other hand, physiologists, cellular, and molecular biologists have often operated outside of evolutionary theory; motivated to understand "how things work" as opposed to why they work one way instead of another (Ghalambor *et al.* 2015). This has led to reductionist approaches which reduce the complexity of interactions occurring within the whole organism or with the external environment (Cohen *et al.* 2012; Martin *et al.* 2011, 2015ner) and prevented thinking about biological pathways in an evolutionary context (Soyer 2012). But now, the fields are converging and there are an increasing number of studies demonstrating how understanding the molecular mechanisms underlying trait correlations, trade-offs, and the related networks (*i.e.* the shared molecular pathways) provides critical insights into the targets of selection and the degree to which selection can or cannot break trade-offs (Chen and Lübberstedt 2010; Schwartz and

Bronikowski 2011; Aubin-Horth *et al.* 2012; Schwartz and Bronikowski 2013; Aubin-Horth 2016; Saltz *et al.* 2017). Our point here is that a focus on understanding the pleiotropic consequences of shared molecular pathways can facilitate integrative biologists to use a similar evolutionary framework across levels of biological organization (*e.g.* Mykles *et al.* 2010; Soyer 2012). This shared perspective will be rooted in network and systems thinking and will emphasize how genomes, molecules, and biological pathways interact to generate the molecular architecture of trait correlations and PRNs and how these networks bias evolutionary responses to natural selection (Wagner *et al.* 2007; Stone and Ayroles 2009; Martin *et al.* 2011; Ciaccio *et al.* 2014; Sommer and Mayer 2015). Our hope is that such approaches will move past correlative evidence for why trade-offs occur and provide a more satisfying explanation for how and why traits are correlated (*e.g.* Wagner 2011; Ihle *et al.* 2015). Most importantly, such an approach allows for *a priori* hypotheses to be generated about the ecological and evolutionary conditions that should result in selection acting at specific points/nodes within the network to overcome trade-offs. For example, one general hypothesis is that evolution to overcome trade-offs should manifest itself by altering downstream components of networks (*e.g.* cis elements or local rQTLs in terms of genetics and receptors in terms of PRNS; Pavlicev and Wagner 2012; Pavlicev and Cheverud 2015; Di Poi *et al.* 2016). One compelling example of this is Pavlicev & Wagner's (2012) *Selection, Pleiotropy, Compensation (SPC) Model*. This model suggests that directional selection will select for beneficial mutations even if these mutations have negative pleiotropic effects on fitness in other traits, because compensatory evolution in rQTLs (QTLs that act on relationships between traits) will correct for the introduced trade-off. Under this model, increased network complexity could potentially be beneficial as more "nodes" in a network would mean more opportunity and more specificity for compensatory changes. Models like the SPC are

intriguing and highlight how network architecture determines whether mutations lead to trade-offs or not.

Lastly, while we advocate for the study of molecular pathways as a way to facilitate integrative thinking about the genome to phenome map, we end on a cautionary note on the limitations and challenges that must be overcome. First, despite biological pathways being highly conserved across taxa, the multi-nodal networks these pathways participate in are highly plastic and offer numerous paths by which the same outcome can be reached (Tononi *et al.* 1999; Motegi and Seydoux 2013; Kafri *et al.* 2016). For example, while we have a very good understanding of the molecular mechanisms and pathways that lead from genes to shaping muscle phenotypes, as well as the selection pressures acting on these mechanisms, Hoppeler (2016) comments that modifications to the structure and function of muscles "can be achieved by an almost unlimited combination of inputs and downstream signaling events". Thus, which pathways are used to shape muscle phenotypes of different species and across diverse environments is difficult to predict, but will certainly differ from those found in model organisms (Hoppeler 2016). In this regard, muscle phenotypes are not likely to be different than any other phenotype in that a common feature of all complex networks is redundancy in parts of the system and robustness to maintain function in response to genetic or environmental perturbations (*e.g.* Masel and Siegal 2009; Matias Rodrigues and Wagner 2009). Thus, proper comparative studies are likely to be very insightful in terms of identifying the various ways in which selection and phylogenetic history shape network architecture. Second, the pleiotropic nature of the genome to phenome map means that the behavior of complex networks will be highly context dependent and sensitive to the internal and external environment of the organism (*e.g.* Zhu *et al.* 2004; Papin *et al.* 2005; Abraham 2008; Oberhardt *et al.* 2009; Zhernakova *et al.* 2017). Similarly, the

fitness consequences of trait correlations due to shared pathways will be dependent on the ecological context the organism experiences (*e.g.* Aubin-Horth *et al.* 2012, 2015; Di Poi *et al.* 2016). For example, the fitness consequences of the pleiotropic melanocortin system depends on the fluctuating selection pressures over time and space for coloration, aggression, and life history (see above). Similarly, a trade-off between osmoregulation and aggression due to the pleiotropic effects of hormones is predicted to only have negative fitness consequences when fish are challenged by salinity and competitive interactions (see above and Figure 2). The context dependency of these fitness trade-offs should lead to strong selection for compensatory changes within the networks (*e.g.* Pavlicev & Wagner 2012), however, in fluctuating environments the cost of these compensatory changes may outweigh the benefits. We are still in the infancy of documenting how much variation exists in the networks and pathways among individuals, populations, and species, and know even less about the consequences of this variation for natural populations. Nevertheless, advances in systems/network biology, molecular biology, and 'omics suggest a bright future for advancing our knowledge of how and when trade-offs are resolved in natural populations.

FIGURES

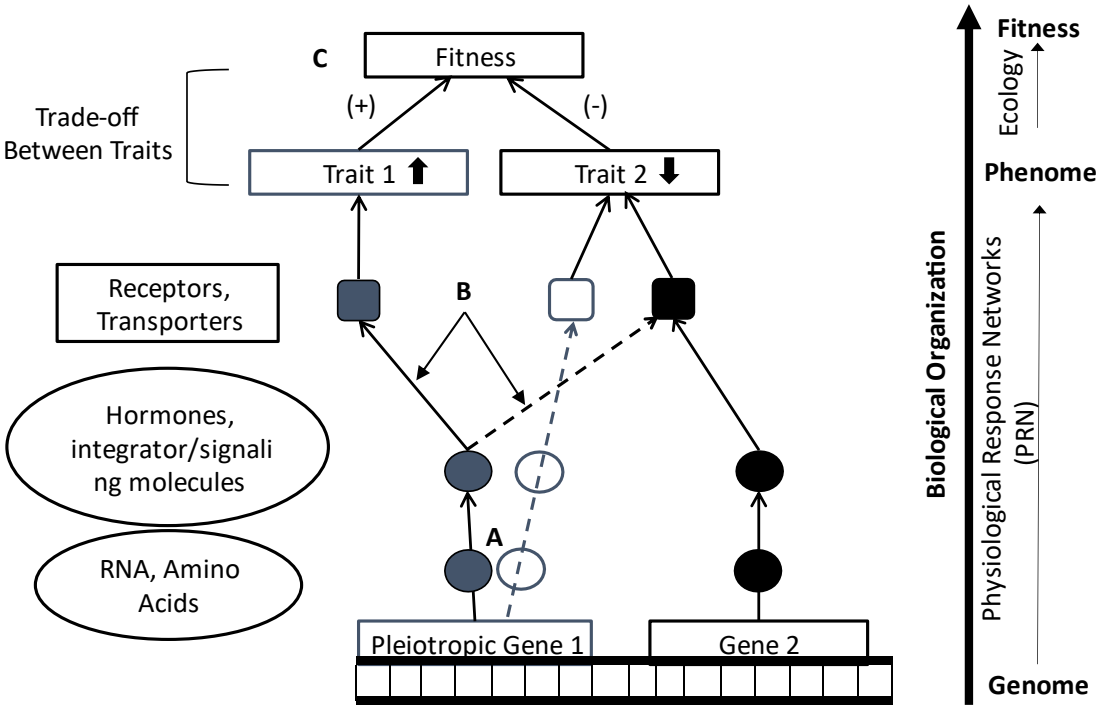

Figure 1.1 A simplified conceptual model of trade-offs in a genome to phenome context. The figure demonstrates two non-mutually exclusive ways in which a trade-off can arise via pleiotropy. (A) denotes how "genetic pleiotropy" can arise if pleiotropic gene 1 has two molecular functions (compare solid line versus dashed line) that are part of two separate pathways leading to Taits 1 and 2. In this case, the filled and open circles are representative of gene products (mRNA, signaling molecules, hormones) that interact with downstream targets represented by filled and open squares (e.g., receptors). In this case, the consequence of the pleiotropic gene is antagonistic because it increases the value of Trait 1 which increases fitness, while decreasing the value of Trait 2 which lowers fitness. (B) denotes "hormonal pleiotropy," where a single signaling molecule like a hormone (light black circle) binds with two different receptors (filled light black and dark black squares), which in turn also increase Trait 1, while decreasing Trait 2. Because this hormone is ultimately a product of gene 1, both situations could be defined as genetic pleiotropy. This distinction may not be readily recognized in studies that only focus on one level of biological system, that is, genetic systems or the endocrine system. However, if the focus is on the pathways instead, the different paths by which Traits 1 and 2 are influenced by gene 1 (as shown by the dashed and solid lines) become evident. (C) denotes the fitness consequence of antagonistic pleiotropy, where increasing Trait 1 increases fitness (e.g., producing more offspring) and decreasing Trait 2 reduces fitness (e.g., reduced survival). The contrasting fitness consequences of this type of pleiotropy are context dependent and likely to change in response to spatial and temporal environmental variation. Lastly, the location along the genome to phenome to fitness map at which these different pathway components (e.g., genes, gene products, hormones, receptors, traits, and fitness) can be measured is depicted on the right side of the figure. As one progresses along with this map, one also moves up in biological

organization (i.e., cells to tissue to whole organism to organism interactions) and this is depicted alongside the map.

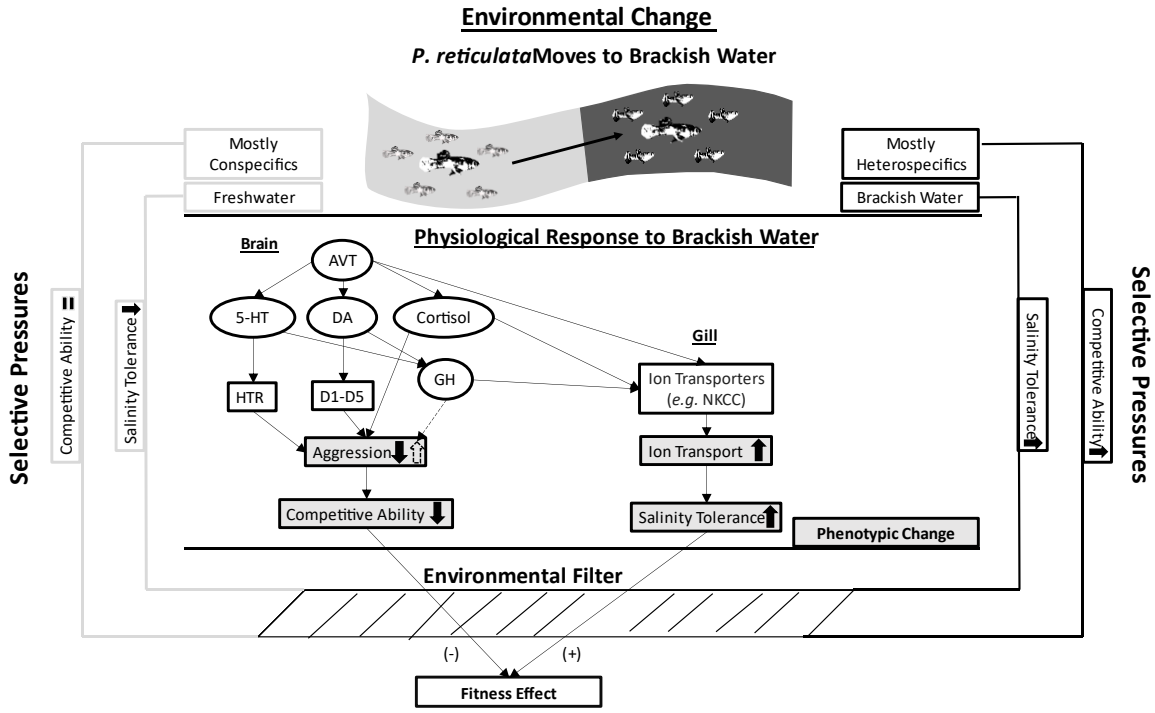

Figure. 1.2 A simplified pictorial summary of a hypothesized trade-off between aggression and salinity tolerance in the ecological context of the guppy, P. reticulata, moving from freshwater to brackish water. If individual guppies were to move from freshwater to brackish water in Trinidad, then they would also encounter swamp guppies, P. picta, in addition to the change in salinity. The osmoregulatory response to the increase in salinity is an increase ion transport in the gills. This response is driven by AVT, cortisol, and GH, which act as integrators or signaling molecules (denoted as circles), and their interactions with receptors/transporters (denoted as squares). These hormones act pleiotropically through shared pathways and lead to a correlation between decreased aggression and increased salinity tolerance. Note that GH is shown to increase aggression, denoted by its connection to the large upward-pointing dashed arrow within the "aggression" filled-square (see text for a more thorough discussion of the pathway). The fitness consequences of these pleiotropic effects are determined by the environmental filter, which suggests guppies cannot simultaneously tolerate increased salinity and cope with the challenge of a closely related competitor.

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CHAPTER 2:

A GENETICALLY BASED ECOLOGICAL TRADE-OFF CONSTRAINS ADAPTIVE EXPANSION AT THE RANGE LIMIT OF THE TRINIDADIAN GUPPY[2]

SUMMARY

The ecological factors that shape geographic range limits and the evolutionary constraints that prevent populations from adaptively evolving beyond these limits remain poorly understood. Here, we investigated why the euryhaline fish, *Poecila reticulata*, is confined to freshwater within its native range, despite being tolerant of brackish water. We hypothesized that competitive interactions with a close relative, *P. picta*, in brackish water prevents *P. reticulata* from colonizing brackish water. Using a combination of field transplant, common garden breeding, and laboratory behavior experiments we find support for the hypothesis that a genetically based trade-off between salinity tolerance and competitive ability plays a significant role in preventing *P. reticulata* from adapting to brackish waters and expanding its geographic range. While genetically based trade-offs are known to act as potential evolutionary constraints, this is one of the first studies to demonstrate their importance in constraining evolution at the range limit in nature.

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INTRODUCTION

A fundamental goal of ecology and evolution is to understand the mechanisms that determine the geographic distributions of organisms. The current limits to species' distributions can largely be explained via hypotheses that test species' eco-physiological responses to abiotic gradients and/or biotic interactions with other species (Holt 2003; Gaston 2009; Sexton et al. 2009; Hargreaves et al. 2014; Louthan et al. 2015; Willi and Van Buskirk 2019). However, to understand the mechanisms preventing populations from *adaptively evolving* and expanding to environments beyond their current range requires an evolutionary explanation with a different set of hypotheses (Futuyma 1998; Willi and Van Buskirk 2019; Angert et al. 2020). Evolutionary hypotheses for the causes of geographic range limits fall under the broader problem of "evolvability", which seeks to understand the constraints on adaptive evolution (Houle 1992; Hansen and Houle 2008; Futuyma 2010; Louthan et al. 2015). Specifically, given enough time and in the absence of any obvious physical barriers to dispersal, what prevents populations from evolving to new environments and expanding their geographic range? In this context, the evolutionary mechanisms underlying range limits are examples of the limits of adaptation in nature, and thus provide "testing grounds" to better understand the adaptive evolutionary process (Sexton et al. 2009).

Evolutionary theory suggests the same factors that can constrain adaptation, such as a lack of genetic variation (e.g. Blows and Hoffmann 2005), the introduction of maladaptive alleles through gene flow (e.g. gene swamping; Tigano and Friesen 2016), and the presence of genetically based trade-offs between traits (Hoffmann and Parsons 1994; Hoffmann and Blows 1994; Roff and Fairbairn 2007; Martin 2015; Mauro and Ghalambor 2020), contribute to observed range limits (Kawecki 2008; Gaston 2009; Willi and Van Buskirk 2019). These factors

are not mutually exclusive, but conclusive support for any of these evolutionary mechanisms as a general explanation for setting range limits remains elusive (Angert et al. 2020). For example, the presence of high amounts of additive genetic variation is commonly found for most traits, and very few empirical examples have found evidence for range limits being set by a lack of genetic variation (e.g. Willi et al. 2006; Eckert et al. 2008; Gould et al. 2014; but see Kellermann et al. 2006). Similarly, there is a paucity of empirical evidence for the role of gene flow introducing maladaptive alleles to populations at the range edge and thus preventing adaptive evolution at a population's range limit (e.g. Sexton et al. 2009; Fitzpatrick et al. 2015; Dennenmoser et al. 2017; but see Pedersen et al. 2017).

Less attention has been given to the role negative genetic correlations and pleiotropy might play in generating fitness trade-offs that prevent adaptive evolution at the range edge (Hoffmann and Blows 1994; Sgrò and Hoffmann 2004; Duffy et al. 2006; Tiffin et al. 2013), despite a large body of research in the ecological literature demonstrating that such fitness trade-offs across environmental gradients are common (Kneitel and Chase 2004; Martin 2015). For example, fitness trade-offs between traits that deal with biotic and abiotic challenges have been described for a wide range of taxa including: heat tolerance and interspecific competition in copepods, fish, birds, and mammals (Chappell 1978; Willett 2010; Martin and Martin 2001; Fausch et al. 1994; Gross and Price 2000), salinity tolerance and competition in fish and plants (Greiner et al. 2001; Polivka 2005; Alcaraz et al. 2008), metabolic plasticity and bacterial defense in beetles (Cioffi et al. 2016) and desiccation tolerance and competition in barnacles (Connell 1961). However, the genetic basis of ecological trade-offs are unknown for all but a few species, preventing evolutionary insights into their importance as a constraint on the evolution which shapes range limits (Anderson et al. 2013; Olsen et al. 2019). Here, we test if an

ecological trade-off between salinity tolerance and interspecific competition determines the range boundary of the Trinidadian guppy (*Poecilia reticulata*) and if so, whether there is a genetic basis to the trade-off.

Study System

The Trinidadian guppy is a euryhaline poecilid fish native to the Caribbean, Central America, and South America and a model system for studying evolution in nature (Reznick et al. 1997; Magurran 2005; Reznick and Travis 2019). Guppies are known as a prime example of rapid adaptive evolution following the colonization of new environments within their native range (Reznick et al. 1997) and have been introduced and become established in streams throughout the world (Deacon et al. 2011). However, in their native habitat of Trinidad, their distributions exhibit an abrupt limit at the freshwater-brackish water boundary across all lowland rivers (Torres-Dowdall et al. 2013; Figure 1). This is an especially dramatic observation because the freshwater boundary point fluctuates daily with the tides and seasonally with the wet/dry season and yet *P. reticulata* is never found in conditions of even 1 psu (practical salinity unit), suggesting that this species behaviorally avoids brackish water (see details below) (Torres-Dowdall et al. 2013). This occurs despite studies showing Trinidadian guppies can tolerate, reproduce in, and evolve improved tolerance to saltwater in the lab (Gibson and Hirst 1955; Shikano and Fujio 1998; Shikano et al. 2001) and persist in brackish, marine, and hypersaline water in parts of their non-native range (Courtenay et al. 1974). Furthermore, this boundary point results in an abrupt shift from high population density to none (Magurran 2005; Torres-Dowdall et al. 2013), despite little change in abiotic features like dissolved oxygen and pH or biotic features like predation (Magurran 2005; Torres-Dowdall et al. 2013). Collectively, these features suggest that the range of *P. reticulata* is not limited due to dispersal, low population

size, predation, or abiotic factors other than salinity. The only other factor that is consistently associated with *P. reticulata's* range limit in addition to salinity is the presence of *Poecilia picta*. *Poecilia picta* is a closely related and similar sized euryhaline poeciliid in lowland Trinidadian rivers. It is found mostly in brackish water, but its range slightly overlaps with *P. reticulata* in freshwater in a pattern repeated across lowland rivers in Trinidad (Torres-Dowdall et al. 2013) (Figure 1). Due to the nature of *P. reticulata's* distribution pattern, we hypothesized that *P. reticulata* avoids brackish water because *P. picta* is competitively dominant under saline conditions. We predicted that this relationship arises because osmoregulation is energetically demanding and pleiotropically linked to aggression (which contributes to competitive ability), and thus the energetic demands and pleiotropic nature of osmoregulation would decrease aggression in saline conditions (e.g. Boeuf and Payan 2001; Tseng and Hwang 2008; Mauro and Ghalambor 2020). Hence, we predicted that the range limit is set by an ecological trade-off between salinity tolerance and competitive ability that is evolutionarily constrained.

In this study we tested several of the ecological and evolutionary assumptions and predictions of our hypothesis that salinity and interspecific competition set the range limit for *P. reticulata*. Specifically, we 1) used a binary choice test to experimentally test if *P. reticulata's* observed abrupt limit at the freshwater-brackish water boundary in Trinidad is behaviorally based, 2) conducted a growth experiment to assess the extent to which salinity exposure increases energetic costs by measuring changes in growth, 3) conducted a common garden experiment in which full-sibling family lines were raised under different combinations of salinity (freshwater vs brackish water) and competitive environment (alone or with *P. picta*) to test if there is a genetic basis to the trade-off between salinity tolerance and competitive ability, 4) carried out a field transplant experiment to investigate the effects of salinity and competition on

survival in nature, and 5) used a lab experiment to investigate if the mechanism by which salinity and interspecific competition reduce fitness is through changes in behavioral dominance. The results of these experiments build upon each other, and collectively provide insight into the ecological and evolutionary mechanisms underlying the range limit.

METHODS

Behavioral avoidance of brackish water in *Poecilia reticulata*.

To test if *P. reticulata's* absence in brackish water is due to behavioral avoidance, we measured the salinity preference (brackish water vs. freshwater) of 33 wild-caught *P. reticulata* (collected from the Caroni river in March 2011) using a "Y-maze" binary choice design (Barnett 1977). This experiment gave fish the option to swim either toward brackish water or freshwater collected from the river from which the fish were collected. The largest difference between these water sources was the salinity (0–0.23psu versus 17–23psu). Variation in other physicochemical variables was not as pronounced as variation in salinity (Freshwater: Temperature=28.2°C, Dissolved Oxygen=42%, pH=7.75; Brackish water: Temperature=29.6°C, Dissolved Oxygen=51%, pH=7.19). Each trial consisted of placing an individual in the base arm of the Y-maze filled with freshwater for a 10-minute acclimation period. After this period, water was simultaneously released into the two response arms, one with freshwater and the other with brackish water (side selected randomly), and a drain in the base arm maintained a linear flow from the response to the base arm (taking advantage of the natural tendency of fish to swim against currents). We considered a preference to have been made when a fish left the base arm and stayed in the selected arm for at least two minutes (see Fig. S1 for a schematic of the Y-maze experiment). We then used a Pearson's X^2 test to determine if fish preferred one salinity over the other.

Effect of salinity and food on juvenile growth rate

To test the assumption that exposure to brackish water results in elevated energetic costs associated with osmoregulation, we conducted a common garden experiment to test how salinity and amount of food influenced individual growth rate. We collected 20 gravid female *P. reticulata* from the Caroni River in 2009 and transported them to Colorado State University where they were individually housed in 10-liter tanks and kept on a 12:12hr light cycle at 25 ± 1ºC. We propagated fish for two generations in the lab to minimize any maternal or environmental effects (following Reznick 1982). Second generation (G2) family lines were generated by randomly crossing lab-born fish within each generation. First generation (G1) litters were split into the two salinity levels used in this study (0psu and 20psu), thus experimental G2 fish were born at the salinity level in which they were tested. Within 24 hours post parturition, three G2 siblings per litter were weighed and each assigned to one of three food levels. The middle food level was equivalent to that used in Reznick (1982) and had been demonstrated to result in positive growth of juveniles (specifically, 10 mg of a paste consisting of equal parts tropical fish flakes and water was given daily to fish by injecting it into their tank with a syringe). For the two other food levels we provided twice as much or half as much as the middle food level. Thus, a minimum of six fish represented each family line, each assigned to one of six possible treatments resulting from the combination of two salinity levels (0 psu and 20 psu) and three food levels (low, medium, high). We estimated growth rate by weighing fish at birth and then again 28 days later when they were four weeks old. We measured specific growth rate as $SGR=(LN (Mass_{week\text{-}4}*Mass_{birth}^{-1})*days^{-1}*100)$(Lugert et al. 2016). We analyzed the data using a mixed model ANOVA with SGR as the response, salinity and food levels and their interaction as fixed effects, and family as a random effect using the package lme4 (Douglas et al.

2015). Least-squared means contrasts between treatments were two-tailed with a Tukey adjustment for multiple tests using the lsmeans package (Lenth 2016). Analyses were done in R (R Team 2019).

Genetic basis of trade-off between salinity tolerance and competitive ability

To test if the trade-off between salinity tolerance and competitive ability with *P. picta* in *P. reticulata* is genetically based, we measured juvenile growth rate in G2s utilizing a split-sibling design. These fish were from the same stock and raised in the same manner as those from the food level experiment, hence G2s were born into the salinity they were tested at and moved to experimental conditions within 24 hours post-parturition. All *P. picta* used in this study were G2s bred from wild caught fish collected at the same time and from the same location as the *P. reticulata* and raised in the same manner. Our two treatments were a salinity tolerance treatment consisting of *P. reticulata* raised by themselves at our low food level in 20 psu and an interspecific competition treatment consisting of *P. reticulata* raised in freshwater with a single G2 *P. picta* competitor at a high food level. We used a low food level for the salinity treatment because the results of the food level experiment showed that the effects of salinity on growth rate are most pronounced in a low food environment (Fig. 2). We used a high food level in the competition treatment to try to ensure that the challenge was solely due to competition and not a general lack of food. In total, 10 family lines and 48 *P. reticulata* fish were used. We used a mixed model ANOVA in R (Team 2019) using the package lme4 (Douglas et al. 2015) with treatment as a fixed effect and family as a random effect to test for a difference in growth rate between the two treatments. We used a Pearson correlation rank test to test for a genetically based trade-off between the two treatments.

Effects of field conditions on trade-off using an enclosure translocation

To test if a trade-off between salinity tolerance and competitive ability reduces *P. reticulata* fitness beyond their freshwater distribution in nature, we conducted a short-term transplant experiment in Trinidad. We set up experimental enclosures at a brackish site (where only *P. picta* naturally occurs) on the Madame Espagnol River (salinity 15–20 psu) and at a freshwater site (where only *P. reticulata* naturally occurs) on the Guayamare River (salinity < 0.5 psu). Within each site we compared the rate of survival over a one-week period. At each site, we had two treatments: one treatment simulated an established population subject only to intraspecific interactions (100% conspecifics; *P. reticulata*), and the other simulated an invasion by *P. reticulata* into a habitat occupied by *P. picta* and the ensuing interspecific interactions (25% conspecific *P. reticulata*, 75% heterospecific *P. picta*). These two treatments were replicated six times per salinity/site (2x2x6=24 enclosures). We placed a total of eight fish per cylindrical mesh enclosures (6.5 liters; diameter=18cm, height=25cm, water able to flow in and out of enclosures), approximately the natural density at these sites (personal observations). The fish in the experiment were *P. reticulata* and *P. picta* collected from a freshwater site along the Guayamare River and *P. picta* collected from a brackish water site at the Madame Espagnol River (15-20psu). Prior to being placed in the enclosures, fish were individually marked using elastomer implants (Northwest Marine Technologies, Inc.). Each fish was randomly assigned to one competition treatment and one salinity treatment. *Poecilia reticulata* that were assigned to the brackish water site were gradually acclimatized to the target salinity by increasing the salinity of their tank by 3 psu per day until reaching a salinity of 15 psu. All *P. picta* were housed in their home salinity before the experiment (0 psu or 15 psu). All fish (regardless of salinity treatment) were held in laboratory tanks for 7-8 days between being captured and starting the experiment.

The effect of competition and salinity on survival was analyzed using a generalized linear model ANOVA test with competition, salinity, and their interaction as fixed effects and log transformed starting mass as a covariate. The analysis was done in R (R Team 2019) using the glm function with a logit link function from the lme4 package (Douglas et al. 2015).

Effect of salinity on behavioral dominance

To test the mechanism by which the salinity alters competitive ability, we conducted an experiment to test if salinity altered dominance relationships between adult male *P. reticulata* and *P. picta*. Differences in aggressive behavior are a way to assess social dominance in fish (Gilmour et al. 2005), hence we analyzed the difference in aggressive behaviors between *P. reticulata/P. picta* pairs in freshwater tanks (0 psu, n=7) and brackish water tanks (15 psu, n=6) over the course of a week. The *P. reticulata* used in this experiment were G2 descendants of fish taken from the same portion of the Caroni river as in previous experiments (just in June 2016). The wild-caught fish were transported to Colorado State University and housed in 1.5 L tanks on large recirculating systems (Ghalambor et al. 2015) but were otherwise bred and raised in the same manner as all previous fish. To serve as competitors to *P. reticulata* in the 15 psu treatment, we used G2 *P. picta* that were descendants of fish taken from a brackish portion of the Caroni river that varied from 25 psu to 35 psu salinity in June 2016. These fish were kept at a salinity of 30 psu but were otherwise housed and bred in the same fashion as other fish. To serve as competitors to *P. reticulata* in 0 psu, we used *P. picta* taken from the same freshwater site as the *P. reticulata* population in June 2016. They were bred and housed in the same manner as described for *P. reticulata*. However, the fish used were either wild caught, G1, or G2 individuals. We did not detect behavioral differences between freshwater *P. picta* of different generations, but we did not have proper replication to formally test this potentially confounding

variable. However, previous behavioral studies on *P. reticulata* from Trinidad found no difference in behavior due to generation in the lab (Gorlick 1976; Seghers and Magurran 1991).

Our experiment began by transferring fish to 10-gallon experimental tanks from the tanks they were housed in. Therefore, both the *P. reticulata* and the *P. picta* in the 15 psu competition tanks experienced an abrupt salinity transfer of 15 psu to begin the experiment (+15psu for the *P. reticulata* and -15psu for the *P. picta;* the *P. reticulata* and the *P. picta* in the 0 psu competition tanks did not experience a salinity transfer because they were housed in 0 psu). During the study, the fish were fed food equivalent to the "low" amount described in the juvenile growth rate experiment. Food was provided to each pair by placing it on a single side of a small dice-sized square. This localized their access to food and encouraged interaction between the fish (personal communication with Dale Broder). We observed each pair of fish for a 5-minute period directly after feeding on days 1, 2, 3, 4 ,7 and recorded aggressive behaviors. Specifically, we recorded chases, nips, and guarding/monopolizing the food source and defined these behaviors the same as they were defined in Seghers & Magurran (1991) (see ethogram in SI). We analyzed the difference in total aggressive behaviors between all *P. reticulata* and *P. picta* pairs using a repeated measures mixed model ANOVA with salinity as a fixed factor and fish ID as a random factor using the package lme4 (Douglas et al. 2015) in R (R Team 2019). We also tested for the effect of body size difference between pairs of fish on aggression by plotting body mass differences between the fish against aggressive behavior difference.

RESULTS

P. reticulata behaviorally avoid brackish water in Y-maze experiment

We found wild-caught *P. reticulata* actively avoid brackish water. Fish placed inside the Y-maze experiment exhibited a statistically significant preference for freshwater and an

avoidance of brackish water ($Probability_{(choosing\ freshwater)}$=0.76, standard deviation= 0.07, Pearson's c^2=8.77, *P*=0.03), independent of the sex of the fish tested (c^2=0.02, *P*=0.9).

Salinity impacts growth rate, but not at high food levels

We found *P. reticulata* had reduced growth rate in brackish water compared to freshwater (Salinity effect: *P*<0.0001; Fig. 2; SI Table S1). As expected, growth rate decreased as food level decreased, but the change in growth rate depended on the salinity (Food level effect: *P*<0.0001; Food level x Salinity: *P*=0.013; SI Table S1). Specifically, at the highest food level, salinity had no significant effect on growth rate (lsmeans test: *P*=0.097; SI Table S2), but compared to freshwater, growth rate of individuals in brackish water exhibited a greater decrease as food level decreased (Fig. 2).

A negative genetic correlation between salinity tolerance and competitive ability

Overall, *P. reticulata* grew faster in the interspecific competition treatment (with *P. picta* in freshwater) than in the salinity treatment (Fig. 3; *P*<0.001; SI Table S3). Importantly, there was a change in the rank order of families between salinity tolerance and competitive ability, where family lines that grew best in the salinity treatment tended to have lower growth rate in the interspecific competition treatment (rho= -0.64, *P*=0.054; SI Table S4).

Salinity and interspecific interactions affect survival in nature

We found that *P. reticulata* survival in the transplant experiment was significantly affected by salinity (*P*=0.004) and the interaction between salinity and competition type (*P*=0.002), whereas competition alone did not affect survival (*P*=0.230) (see SI Table S5). Specifically, when comparing treatments reflecting salinity transitions under both competition types, we found that *P. reticulata* survival was reduced in brackish water compared to freshwater

under the interspecific competition treatment (P=0.0164) but not under the intraspecific competition treatment (P=0.2508) (SI Table S6) (Fig. 4).

Salinity alters interspecific dominance relationships

We found that aggressive behaviors were similar between the two species in freshwater, but that *P. picta* exhibited significantly more aggressive behaviors than its *P. reticulata* competitor in brackish water (P=0.035; Fig. 5; SI Table 7). Further, we found that there was no relationship between difference in body mass between pairs of fish and difference in aggressive behaviors (Fig. S2).

DISCUSSION

What factors shape geographic range limits and what prevents populations from adaptively evolving and expanding their ranges? Answering this question has proven exceedingly difficult for most species because it not only requires understanding the mechanisms for contemporary distributions (Gaston 2009; Sexton et al. 2009; Louthan et al. 2015; Willi and Van Buskirk 2019), but also an understanding of the factors that constrain adaptive evolution (Hoffmann and Parsons 1994; Hoffmann and Blows 1994; Wagner and Altenberg 1996; Futuyma 2010; Payne and Wagner 2019; Angert et al. 2020). Here, we asked the general question: why is the distributional limit of *P. reticulata* associated with freshwater, despite being euryhaline and tolerant of brackish water? Using a combination of field and lab experiments, we find support for the hypothesis that *P. reticulata* behaviorally avoids brackish water because it goes from being an equal competitor with its close relative *P. picta* in freshwater to being behaviorally subordinate in brackish water. Furthermore, we find evidence that increased salinity

tolerance in *P. reticulata* exhibits a negative genetic correlation with competitive ability with *P. picta* in freshwater, suggesting a constraint on the evolution of increased salinity tolerance.

Our investigation began with the simple field observation that *P. reticulata* is never found in brackish water despite the freshwater-brackish water boundary fluctuating daily and seasonally (Torres-Dowdall et al. 2013). This pattern suggested that *P. reticulata* can detect low levels of salinity and behaviorally avoid them. In our salinity preference experiment we found *P. reticulata* exhibits a strong preference for freshwater over brackish water. Behavioral avoidance and reduced dispersal beyond the range limit has been suggested to evolve when there is a predictable fitness cost to encountering the avoided environment (Holt 2003; Duckworth 2009) and we found evidence for such costs in this study. We found that while *P. reticulata* is tolerant of moderate levels of salinity, there appears to be an elevated energetic cost of osmoregulation that is mediated by food availability. This is consistent with previous work on the energetics of osmoregulation (see Boeuf and Payan 2001; Tseng and Hwang 2008)(Fig. 2). Specifically, we found juvenile growth rate declined in brackish water compared to freshwater but only at medium and low food levels (Fig. 2). These results suggest that the elevated osmoregulatory costs only manifest themselves when access to food is limited, but in the lowland rivers of Trinidad where freshwater transitions to brackish water, there are no obvious changes suggestive of drastically reduced food availability. Such results suggest that any fitness costs due to food limitation in nature would have to arise through interference competition with *P. picta,* as is observed in many contexts where closely related species replace each other along environmental gradients (see Martin 2015). Indeed, salinity often predicts aquatic species distributions (e.g. Kefford et al. 2004; Kefford et al. 2012), but the degree to which salinity acts in conjunction with biotic factors, such as competition, to shape distributions remains largely unexplored in the

ecological and physiological literature. We tested whether the nature of competition changes between freshwater and brackish water by measuring the survival of *P. reticulata* in freshwater and brackish water enclosures where *P. reticulata* interacted with conspecifics or *P. picta*. We found that *P. reticulata* survival was most reduced when fish were in brackish water with *P. picta* (Fig. 4) and that the interaction between salinity and competition type was significant (SI table 5), suggesting that it is the combination of brackish water and competition with *P. picta* that reduces fitness and not either factor alone. However, we were unable to observe the behavior of the fish inside the enclosures and could not evaluate the mechanism by which *P. picta* reduces the fitness of *P. reticulata*. However, our laboratory-based competition experiments between *P. reticulata* and *P. picta* suggest this mechanism is mediated through interference competition as *P. reticulata* exhibited fewer aggressive behaviors than *P. picta* competitors in brackish water, but a similar number of aggressive behaviors in freshwater (Fig. 5). Alcaraz et al. (2008) arrived to a similar conclusion when finding that salinity mediated competitive interactions between an invasive mosquitofish (*Gambusia holbrooki*) and a native cyprinodont (*Aphanius fasciatus*). The trade-off between the ability to tolerate salinity and maintain aggressive behaviors among fish may be a general pattern, as the same hormones involved in osmoregulation have pleiotropic effects on the serotonergic, dopaminergic, and cortisol pathways that control aggression (Mauro and Ghalambor 2020). This connection has also been observed in studies showing fish that become subordinate within social hierarchies (as measured by differences in aggressive behaviors, among other methods) have elevated cortisol levels and greater difficulty in regulating ions (Gilmour et al. 2005; Jeffrey et al. 2014), suggesting the link between osmoregulation and aggression could be a general pattern.

Trade-offs between different functions underlie many ecological theories explaining patterns of species diversity (Jessup and Bohanna 2008; Martin 2015; Clark et al. 2018), yet the evolutionary maintenance of such trade-offs remains an open question (Anderson et al. 2013). By comparing growth rates of full-siblings reared in brackish water alone versus in freshwater with *P. picta*, we found that the families that had higher growth rate under brackish conditions, tended to have lower growth rate when interacting with *P. picta* in freshwater (Fig. 3). Such results suggest a genetically based trade-off and that evolving increased salinity tolerance could come at the expense of competitive ability, at least in freshwater but possibly in brackish water as well. Given that *P. reticulata* is subordinate to *P. picta* in brackish water but not in freshwater (Fig. 5), *P. reticulata's* competitive ability in freshwater could be eroded by a correlated response to selection for improved salinity tolerance.

Pleiotropy or linkage could give rise to the negative genetic correlation between competitive ability and salinity tolerance and constrain evolution to expand beyond the range limit (e.g. Duffy et al. 2006), but other non-mutually exclusive mechanisms could also constrain evolution and expansion of *P. reticulata* into brackish waters as proposed by Willi and Van Buskirk (2019). First, based on extensive sampling over time and space (Magurran 2005; Torres-Dowdall 2013), it is unlikely *P. reticulata* is dispersal limited at its range edge. Rather our results argue that *P. reticulata*, like many other organisms, is instead range limited due to a lack of adaptation to the environment beyond its current range (Hargreaves et al. 2014b). Second, a common hypothesis for lack of adaptive evolution is a lack of genetic variation (Blows and Hoffmann 2005). Yet this is unlikely in the case of *P. reticulata* because near the freshwater/brackish water transition zone population sizes are very large (Magurran, 2005; personal observations), certainly much larger than populations of this species that have exhibited

rapid parallel adaptation in nature elsewhere in Trinidad (e.g. Reznick et al. 1997; Reznick and Travis 2019), and because we found among family variation in salinity tolerance (Fig. 3). Moreover, as discussed in the introduction, very few empirical studies have found evidence for range-edge populations lacking enough genetic variation to constrain evolution (Willi et al. 2006; Eckert et al. 2008; Gould et al. 2014). Third, gene flow from large central populations could prevent the fixation of adaptive alleles in the population at the range limit (Lenormand 2002; Gaston 2009; Tigano and Friesen 2016). It seems likely that all gene flow into *P. reticulata's* freshwater edge populations is coming from populations that rarely experience brackish water (see Fig. 1). This sets-up a scenario in which this type of gene swamping could be prominent in our system. However, gene swamping is more likely when the population providing the "maladaptive migrants" is larger than the recipient population (Gaston 2009). There is an overall lack of evidence that edge populations are smaller than central ones (Dallas et al. 2017) and *P. reticulata* in Trinidad are known to have large populations near their freshwater range limit (Magurran 2005). Additionally, adaptive divergence has been observed to evolve among *P. reticulata* populations experiencing substantial levels of gene flow over short spatial distances (Torres Dowdall et al. 2012; Fitzpatrick et al. 2015). Nevertheless, given the lack of any barriers to movement we cannot discount a role for gene swamping in constraining evolutionary change at the range margin.

Overall, our results suggest that a genetically based trade-off between salinity tolerance and interspecific competitive ability contributes to *P. reticulata* being restricted to freshwater in Trinidad. While negative genetic correlations are well known to act as potential evolutionary constraints (e.g. Hansen and Houle 2008; Hughes and Leips 2018), the relative importance of such correlations in constraining evolution at range limits has received very little attention in

animals, partly because of the difficulty of conducting experiments on natural populations. However, our work here demonstrates how a combination of field and lab studies commonly used in plant studies (e.g. Anderson et al. 2013), can be applied to animals to better understand the ecological and evolutionary determinants of range limits. Lastly, the interaction between salinity and behavior described here suggests that predicting the biological consequences of salinization of freshwater environments may be more complicated than simply measuring salinity alone (Ghalambor et al. 2021). This is a particularly important challenge given that anthropogenic climate change is predicted to increase sea level and the salinity levels of coastal rivers and estuaries (Rice et al. 2012; Ghalambor et al. 2021).

FIGURES

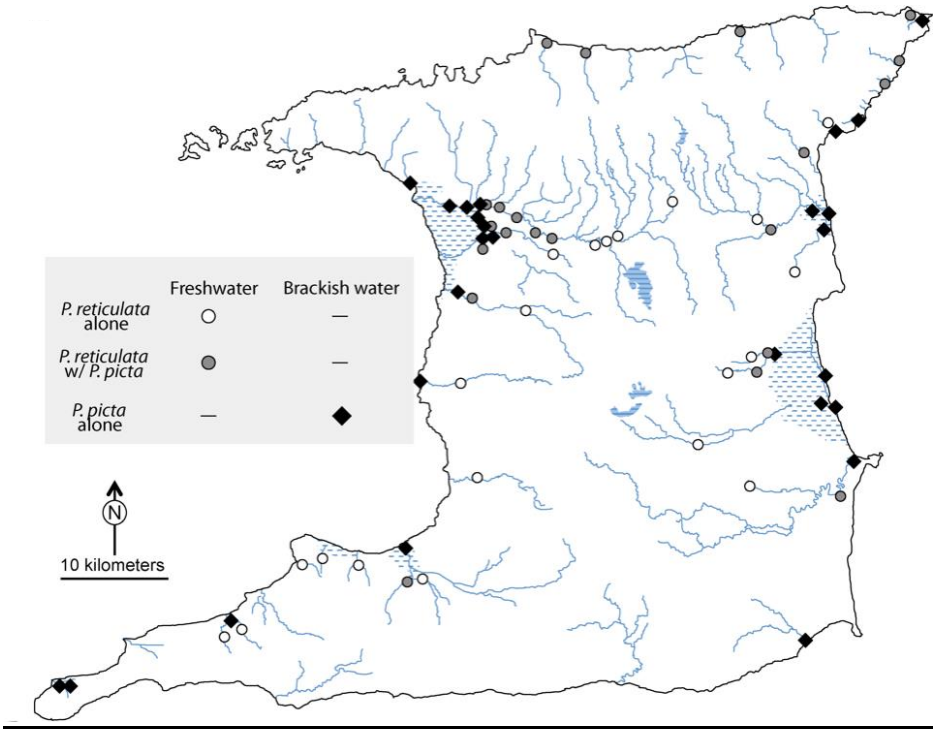

Figure 2.1: The distribution of *P. reticulata* and *P. picta* in the coastal rivers along the eastern and western coasts of Trinidad (adapted from Torres-Dowdall et al. 2013). Open circles represent freshwater sites where only *P. reticulata* has been found, grey circles represent freshwater sites where both species have been found together, and black diamonds represent brackish sites where only *P. picta* have been found. In all the rivers sampled, *P. reticulata* and *P. picta* only overlap in freshwater and *P. reticulata* is never found in brackish water.

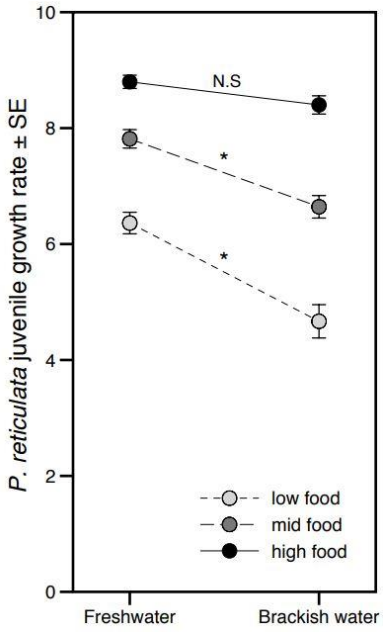

Figure 2.2: Salinity and lower food levels have a negative effect on the growth rate of *P. reticulata.* The negative effects of salinity are only evident at the lower food levels as no difference in growth rate was observed between fish reared in brackish and freshwater at the highest food level (denoted by "N.S"). There were, however, significant differences between the salinity treatments at the other food levels (denoted by "*") (least-squares mean differences with Tukey HSD adjustment). Means +/- SEs are displayed.

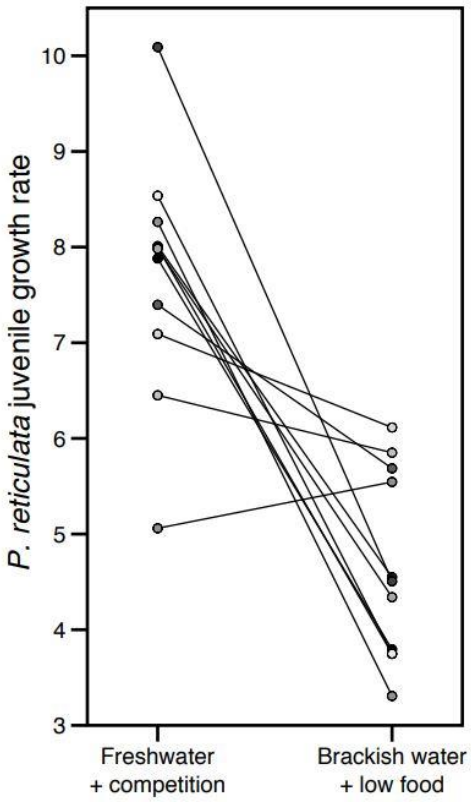

Figure 2.3: Juvenile growth rate of *P. reticulata* family lines with siblings split into either a salinity tolerance (brackish water + low food alone) or a competition (freshwater + competition with *P. picta*) treatment. Ends of lines represent family mean growth rates and lines connect family means across treatments. Fish grew significantly better in the competition treatment than the salinity treatment and families that grew relatively better in one treatment tended to grow significantly worse in the other treatment.

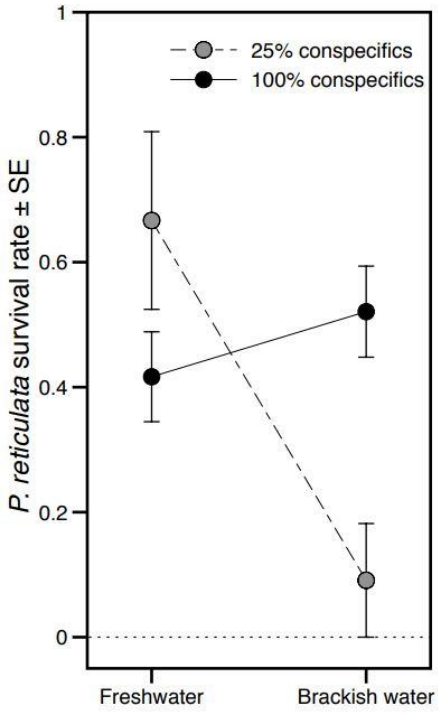

Figure 2.4: Survival rate over the course of one week for *P. reticulata* that were placed in enclosures in rivers in Trinidad. The enclosures were either in freshwater or brackish water sites and either contained only other *P. reticulata* (black, solid line) or 75% P. *picta*/25% *P. reticulata* (grey, dashed lin). Means +/- SEs are displayed.

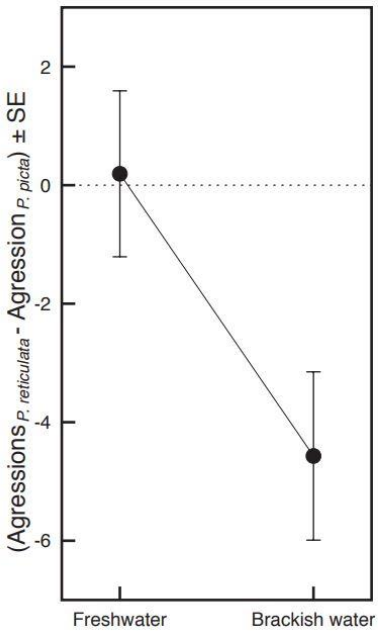

Figure 2.5: The difference in aggressive behaviors between a *P. reticulata* and a *P. picta* in either a freshwater or brackish water competition tank. Hence, positive values indicate that *P. reticulata* was dominant and negative values indicate that *P. reticulata* was subordinate. *P. reticulata* was neither dominant nor subordinate to *P. picta* in freshwater but was subordinate to *P. picta* in brackish water. Means +/- SEs are displayed.

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CHAPTER 3:

AN INVESTIGATION INTO POTENTIAL PHYSIOLOGICAL MECHANISMS UNDERLYING A NICHE CONSTRAINING ECOLOGICAL TRADE-OFF[3]

SUMMARY

Understanding the mechanisms which constrain niche evolution remains an unresolved challenge in attempts to explain why populations fail to expand beyond the set of environmental conditions they occupy. Because organisms are highly integrated systems, pleiotropy between different traits and functions represent one type of ecological and evolutionary constraint on niche expansion. Numerous field and laboratory studies have demonstrated fitness trade-offs across different environments, but few studies have demonstrated why trade-offs occur and why they persist as a constraint on niche expansion. Previous research has shown *Poecilia reticulata* exhibit a trade-off between salinity tolerance and competitive ability with a close relative (*Poecilia picta*), and this trade-off is hypothesized to prevent niche expansion into brackish water. Here, we begin to investigate if pleiotropy in physiological networks prevents natural populations of the euryhaline fish *P. reticulata* from expanding their ecological niche. We first synthesize previous work on the physiology underlying osmoregulation and aggression and propose a potential network that could underly a trade-off between those traits. We next look for empirical support for the network and trade-off by simulating a brackish water invasion in the lab by exposing *P. reticulata* to a salinity challenge in the presence and absence of *P. picta* and measuring transcriptomic responses in the brain. We find that the salinity challenge resulted in *P.*

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reticulata losing body mass and becoming less aggressive when they were in the presence of *P. picta*, but not in the presence of conspecifics. We identified 1675 genes that were differentially expressed in response to the brackish water invasion and organized into 13 modules of co-expressed genes. Of these modules, a module of 422 genes and a module of 155 genes were associated with a decrease in aggression. We found evidence suggesting that this negative relationship arises because salinity exposure results in activation of hormonally mediated pathways in the brain associated with ion regulation and aggression. Overall, we show how overlapping biological pathways have the potential to explain why ecological trade-offs are observed.

INTRODUCTION

A fundamental question at the interface of ecology, evolution, and physiology is to understand the mechanisms that constrain populations from expanding their ecological niche (Arnold 1992; Chase and Leibold 2003; Futuyma 2010). Expansion and colonization into new environments can result in strong directional selection and rapid adaption in natural populations (i.e. Endler 1986; Reznick et al. 1997; Reznick and Ghalambor 2001; Hendry et al. 2008). However, strong selection can also lead to extirpation, extinction, and failures of populations to adapt to new environments (Bradshaw 1991; Futuyma 2010; Carlson et al. 2014; Bay et al. 2017). Indeed, experimental translocations beyond a population's geographic range limit typically reduce fitness (Hargreaves et al. 2014). A large body of ecological research has shown these reductions in fitness often arise because of trade-offs between ecologically important traits, such as those associated with tolerance to the abiotic environment versus those associated with biotic interactions (Chapter 2; Louthan et al. 2015; Martin 2015). Yet, why ecological trade-offs

occur between seemingly unrelated traits and the potential mechanisms that might underly the trade-off have rarely been explored.

Theory predicts that trade-offs arise because organisms are hierarchically integrated genetic, developmental, and physiological systems (Cohen et al. 2012; Mauro and Ghalambor 2020). Like any complex system dependent on the coordination of multiple interacting parts to carry out specific functions, changes in any one trait or function will impact other traits and functions depending on the architecture or pattern of relationships (Soyer 2012; Martin 2015). Thus, fitness trade-offs arise when a beneficial change in a trait or function has a detrimental impact on other traits or functions (Roff and Fairbairn 2007; Mauro and Ghalambor 2020). Examples of such trade-offs include negative genetic correlations between traits due to linkage and pleiotropy (e,g. Bennett and Lenski 2007; Solovieff et al. 2013; Chen and Zhang 2020), allocation of finite resources to competing traits (e.g. Zera and Harshman 2001; Bourg et al. 2019), and the coordinated expression of multiple traits due to the action of hormones and other signaling molecules (e.g. Cox et al. 2016; Dantzer and Swanson 2017). Systems and network approaches that describe the shared biological pathways between traits and functions provide a conceptual framework for understanding the highly integrated complex genetic and physiological responses to the environment (Cohen et al. 2012; Lotterhos et al. 2013; Martin and Cohen 2015). Such approaches can bridge the gap between the phenotypic measures of complex traits and their physiological basis (Wagner et al. 2007; Gove et al. 2012; Martin and Cohen 2015; Stanford et al. 2020), thus providing a foundation upon which the ecological and evolutionary implications of trade-offs can be evaluated (Crombach and Hogeweg 2008; Draghi and Wagner 2009; Mauro and Ghalambor 2020).

We previously demonstrated that the euryhaline Trinidadian guppy (*P. reticulata*) experiences a genetically based trade-off between salinity tolerance and aggression towards the swamp guppy (*P. picta*) and argued that this trade-off explains why *P. reticulata* are absent from brackish water inhabited by swamp guppies (Chapter 2). Here, we 1) formulate a hypothesis for the mechanism underlying the trade-off by synthesizing previous work on the physiological basis of salinity tolerance and aggression & 2) take a network approach to begin to investigate our proposed mechanism.

Synthesis of the Physiology Underlying Aggression and Salinity Tolerance in Teleost Fish

Both salinity tolerance and aggression are complex, polygenic traits in fish with important ecological roles (i.e. osmoregulation and competition respectively) (Santangelo and Bass 2006; Filby et al. 2010; Kultz 2015). Although the physiological basis of both traits has been studied extensively and the two traits are known to interact phenotypically (i.e. Gilmour et al. 2005), we know of no study that has assessed the physiological basis of these two traits simultaneously. We previously hypothesized that the physiological networks underlying aggression and osmoregulation/salinity tolerance share several biological pathways and hence should be correlated (Mauro & Ghalambor 2020). Here, we now formally propose a network depicting those shared pathways (Fig. 1). Somatostatin and arginine vasotocin (AVT) both regulate cortisol and growth hormone which are important for ion excretion and cell proliferation during osmoregulation in brackish/salt water (Lin et al. 2000; Reinecke et al. 2005; Balment et al. 2006; McCormick and Bradshaw 2006; Trainor and Hofmann 2006; Mancera and Mccormick 2007; Kreke and Dietrich 2008; Filby et al. 2010; Kültz D. 2015). However, cortisol is known to decrease aggression in fish (DiBattista et al. 2005; Gilmour et al. 2005), thus this pathway represents a potential trade-off between salinity tolerance and aggression (Fig. 1). Interestingly,

growth hormone is known to increase aggression (Jönsson and Björnsson 2002; Reinecke et al. 2005; Filby et al. 2010) in fish, hence the growth hormone pathway represents a potential avenue to ameliorate the trade-off (Fig. 1). Further, AVT increases both serotonergic and dopaminergic activity (Morando et al. 2009), which both have known effects on aggression. Increased serotonergic activity decreases aggression in fish via the activation of 5-HT receptors (Filby et al. 2010). Dopamine can have a direct, receptor-dependent effect on aggression (d3 receptors decrease aggression whereas d2 receptors increase aggression) and an indirect increasing effect on aggression by stimulating the release of growth hormone (Björnsson et al. 2002; Kreke and Dietrich 2008; Filby et al. 2010). Hence, AVT's effect on serotonin and dopamine represent more pathways that could either underly or ameliorate the trade-off (Fig. 1). Lastly, osmoregulation and aggression are energetically intensive processes, hence the activation of metabolic pathways can increase performance in both traits (Tseng and Hwang 2008; Filby et al. 2010) (Fig. 1). Overall, we hypothesize that the physiological networks of these two traits overlap in such a manner to create a trade-off.

We investigate this hypothesis using use a network approach to analyze the guppy transcriptome in the context of an experiment that mimics guppies invading brackish water in the presence of conspecifics and their closely related competitor *Poecilia picta*. Specifically, we analyzed guppy behavior, body condition, and gene expression in the brain as guppies competed for food against either an ecological competitor species (*P. picta*) or a conspecific under different salinity conditions.

METHODS

Effect of Salinity and Competition on Body Condition and Behavior

Study fish

To account for maternal and environmental effects, the *P. reticulata* in this study were F2 descendants of fish taken from Trinidad in June 2016. They were taken from a freshwater portion of the Caroni river where *P. reticulata* and *P. picta* are found together. The wild-caught fish were transported to Colorado State University and housed in 1.5L tanks on large recirculating systems (Ghalambor et al. 2015). They were kept on a 12:12hr light cycle under temperature conditions that mimicked Trinidad (25 +/- 1°C) and in freshwater. They were fed in the same fashion as described by Reznick (1982). The F1 and F2 generations were generated by randomly crossing fish to create unique family lines (while avoiding cousin or sibling mating). The *P. picta* used in brackish water treatments were also F2 descendants of fish taken from Trinidad in June 2016; however, they were taken from a brackish portion of the Caroni river that varied from 25psu to 35psu salinity. These fish were kept at a salinity of 30psu but were otherwise housed and bred in the same fashion as described for *P. reticulata*. *P. picta* used in freshwater treatments were taken from the same freshwater site and at either the same time or 1 year later as the *P. reticulata* population. They were bred and housed in the same manner as described for *P. reticulata*. However, the experimental fish used were either wild caught, F1, or F2 individuals (due to a lack of F2 individuals). We did not detect behavioral differences between freshwater *P. picta* of different generations, but we did not have proper replication to formally test this potentially confounding variable. However, previous behavioral studies on *P. reticulata* from Trinidad found no difference in behavior due to generation in the lab (Gorlick 1976; Seghers and Magurran 1991).

Experimental Design

To investigate the effects of salinity, competitive environment, and their interaction on adult behavior and fitness, we measured the change in body condition and the change in

aggressive behavior of adult male *P. reticulata* in a series of experimental tanks. Following a balanced 2x2 factorial design, we varied salinity and competition type: tanks were either 0psu or 15psu and either consisted of a *P. reticulata* with another *P. reticulata* (intraspecific competition) or a *P. reticulata* with a *P. picta* (interspecific competition). Food was provided to each pair/tank by placing 50% of the regular allotment of food (for 2 fish) on a single side of a small dice-sized block. This localized access to food and encouraged interaction between the fish (personal communication with Dale Broder). Because the brackish *P. picta* were housed in 30psu and the *P. retciulata* were housed in 0psu (as described in the previous section), both species of fish experienced an abrupt salinity transfer of 15psu to begin experiments in 15psu experimental tanks. Because the freshwater *P. picta* were housed in 0psu like *P. reticulata*, neither species experienced a salinity change when experiments started in 0psu experimental tanks.

To assess the effect of the experimental environment on body condition in each fish ($n \geq 6$ per treatment), we took length and mass measurements 48hrs prior to the start of the study to serve as a baseline and then at the end of the one-week experiment (we continued this study for a total of 3 weeks on a subset of fish and results did not change, see Tables S3-S4). These measurements were taken by first anesthetizing the fish with a dose of MS-222 and then weighing and photographing the fish (length was derived from the photo using ImageJ). Body condition was calculated from length and mass using the equation suggested by Peig & Green (2010). The difference in body condition at the end of the week was used as the response variable in an ANOVA, with salinity and competition type and their interaction as fixed effects and "start-day" as a covariate (sets of individuals began the experiment on 3 different days to ease the processing on each day). The analysis was conducted in R (R Team 2019) using the lme4 package (Douglas et al. 2015). We also conducted a least-squared means post-hoc

comparison of the two salinity treatments under each competition treatment (using a Tukey adjustment for multiple comparisons) using the lsmeans package (Lenth 2016) in R.

Differences in aggressive behavior are a way to assess social dominance in fish (Gilmour et al. 2005). Hence, to better understand how salinity altered dominance relationships between *P. reticulata* and *P. picta*, we analyzed the difference in aggressive behaviors between each *P. reticulata/P. picta* pair in both salinities. Each pair of fish was observed for a 5-minute period directly after feeding on days 1, 2, 3 ,4 ,7 and aggressive behaviors were recorded. Aggressive behaviors included nips, chases, and monopolizing the food source and were defined in the same ways as defined in Seghers & Magurran (1991) (see ethogram in SI). We analyzed the difference in total aggressive behaviors between all *P. reticulata* and *P. picta* pairs using a repeated measures mixed model ANOVA with salinity as a fixed factor and fish ID as a random factor using the package lme4 (Douglas et al. 2015) in R (R Team 2019).

Effect of Salinity and Competition on Gene Expression

RNA Extraction, Sequencing, & Alignment

To better understand the transcriptomic architectures underlying the potential salinity tolerance/aggression trade-off, a subset of *P. reticulata* were sacrificed after 24hrs under experimental conditions ($n \geq 5$ per treatment) via decapitation followed by a brain pith. Whole brain was then immediately extracted and placed in RNAlater. Brain mRNA was extracted using Qiagen RNeasy Mini Kits. Messenger RNA transcriptome libraries were prepared and sequenced at the University of Texas at Austin's Genetic Sequencing and Analysis Facility (GSAF). 100-base pair single-end sequencing was done via TagSeq protocol (Lohman et al. 2016) using a NovaSeq 6000 platform. Sequencing resulted in 5.36 million sequences per sample (s.d. 1.1 million sequences per sample). We used Trimmomatic (Bolger et al. 2014) to filter the sequencies based on quality. Sequence quality was assessed for each read in 4-bp sliding

windows. Sequences were trimmed when Phred33 quality scores fell below an average of 15 within the window, reads were trimmed by 15bp at the front and back to remove potential adapter contamination, and any reads below 36bp after trimming were dropped. Cleaned reads were aligned to the *P. reticulata* genome (Kunstner et al. 2016) using the Trinity pipeline (Grabherr et al. 2013) with the Kallisto alignment and TPM read count normalization options as suggested by Zhang et al (2017). Alignment resulted in an average of 79% of reads per sample being successfully mapped (s.d. 3.9%).

Gene expression analyses

We used DESeq2 (Love et al. 2014) to perform differential expression analyses on the samples in order to classify biologically important sets of genes. We used the adjusted p-value statistic from DESeq2 and a cutoff of 0.05 as our definition of significant differential expression. We focused on those differentially expressed genes associated with transitioning from freshwater to brackish water by computing two lists of differentially expressed genes. The first list were those genes differentially expressed between the interspecific freshwater and interspecific brackish water treatments and the second list were genes differentially expressed between the intraspecific freshwater and intraspecific brackish water treatments. We then combined the unique elements from these two lists led to create a set of genes broadly associated with invading brackish water under different competition scenarios (brackish water invasion genes).

We used WGCNA (Langfelder and Horvath 2008) to identify co-expressed modules of genes that were associated with changes in aggression and transitioning from freshwater to brackish water. We wanted to limit our analysis to only those genes used when fish entered

brackish water from freshwater, so only the brackish water invasion genes that we identified (see above) were used as input. Using the blockwisemodules function in WGCNA, we created regulatory modules of genes using a soft-thresholding approach (k=10) (Fig. S1) and merged modules that were highly correlated with one another (R^2>0.75). To identify regulatory modules associated with aggression, we looked for correlations between module eigengene values and aggressive behavior counts using a Pearson correlation with the cor function of WGCNA (Langfelder and Horvath 2008). Eigengenes were calculated as the first principal component of regulatory variation for each module.

Next, we identified "integral genes" in each of our modules that were significantly correlated with aggression. Specifically, we followed Horvath and Dong (2008) and defined integral genes as those genes that had a gene significance (absolute value of the correlation between the gene and the trait) value greater > 0.2 & a module membership (correlation between the module eigengene and the gene expression profile) absolute value > 0.8. The selection of these genes is similar to the selection of "hub genes" in other network analyses but was done in a more statistically and biologically relevant manner than most hub gene selection methods which typically only rely on connectivity or degree when identifying genes (Horvath and Dong 2008). That said, module membership significantly correlated with intramodular connectivity for all the modules in which integral genes were identified (Fig. S2), thus we do not predict a dramatically different result from methods that only use connectivity. Although designed for analyses on humans, we ran GenClip3 (Wang et al. 2020) on integral genes in one module (turquoise) to identify key biological pathways within sets of genes within WGCNA modules as has been done in previous network analyses that utilized WGCNA (i.e. Liu et al. 2016). Lastly, we ran Gene Ontology analyses on the modules that were significantly correlated with aggression and on the

integral genes within each of those modules using ShinyGo (Ge et al. 2020) with a corrected FDR significance cut-off of 0.05.

RESULTS

Salinity and interspecific competition interact antagonistically in brackish water.

We found that neither salinity (p=0.095) nor competition type (p=0.256) affected *P. reticulata* change in body condition, but that the interaction of salinity and interspecific competition resulted in a significant decrease in body condition (p=0.048) (Fig. 2, Table S1). Further, we found that guppies facing interspecific competition performed significantly better in freshwater than in brackish water (p=0.0155) whereas there was no difference between salinity groups when guppies faced intraspecific competition (p=0.79) (Table S2).

Salinity alters interspecific dominance relationships.

We found that aggressive behaviors were similar between *P. reticulata* and *P. picta* in freshwater, but that *P. picta* exhibited significantly more aggressive behaviors than its *P. reticulata* partner in brackish water (p=0.035; Fig. 3; Table S5). Further, we found that there was no relationship between difference in body mass between pairs of fish and difference in aggressive behaviors (Fig. S4).

Data processing, identification of brackish water invasion genes, identification of modules correlated with aggression, and identification of integral genes.

After data clean-up and filtering, 30,026 transcripts from 26 brain samples were obtained and used as input for the differential expression analysis. A total of 1675 unique transcripts were found to be differentially expressed between freshwater and brackish water treatments (salinity

transition genes): 288 unique to the intraspecific comparison, 1239 unique to the interspecific treatment, and 148 shared across both comparisons (Table S8).

Using the WGCNA package in R and the brackish water invasion genes as input, we grouped the genes into 13 different modules of co-expressed genes (254 genes were left ungrouped) (Table 1). Four of the 13 modules were significantly correlated with aggressive behaviors: two negatively correlated (turquoise and brown modules) and two positively correlated (red and green modules) (Table 1). This meant that the gene expression profiles of the turquoise and brown modules were significantly correlated with a decrease in aggression whereas the gene expression profile for these red and green modules were significantly correlated with an increase in aggression. The turquoise and brown modules had closely correlated eigengene values and the red and green modules did as well; however, the two groups (turquoise/brown and red\green) were relatively dissimilar (Fig. S3). Integral genes (see methods for definition) were then identified for each module significantly correlated with aggression. The full list of genes (and integral genes if applicable) within each of these modules can be found in Tables S9-S12.

Functional analysis of modules and integral genes: The turquoise module appears to be extremely important to the trade-off.

Using ShinyGo (Ge et al. 2020), we tested for functional enrichment of Biological Processes, Cellular Components, Molecular Function, and KEGG pathways for each module that was significantly correlated with aggression and for each list of integral genes within each significantly correlated module. The full results can be found in supplementary Table S6. We report the most impactful GO results from the integral gene lists of the largest module

significantly associated with a decrease in aggression (turquoise) and largest module significantly associated with an increase in aggression (green) in Table 2. We found that the turquoise module contained genes enriched for GO terms that related to pathways we had identified *a priori* as supporting a salinity tolerance/aggression trade-off (Fig. 1). We also found the turquoise module contained genes enriched for GO terms that related to pathways we had identified *a priori* as a potential way the trade-off could be ameliorated (Fig. 1). The green module contained genes enriched for GO terms relating to cell proliferation, metabolic pathways, and mitochondria function (Fig. 1). Lastly, we ran the "pathway analysis" in GenClip3 (Wang et al. 2020) on the integral genes in the turquoise module to further explore pathways significant to this module (Table S7). The results from the GO analysis were largely corroborated by the pathway analysis. Notably though, the "glucocorticoid regulatory receptor network" pathway was found to be enriched within the turquoise module via GenClip3.

DISCUSSION

The Trinidadian guppy, *P. reticulata*, is a well-known model system for studying rapid adaptation to new environments in nature (Reznick et al. 1997). Yet, at their geographic range limit, guppies have been unable to expand their ecological niche into brackish water and we previously found support that a trade-off between salinity tolerance and competition with a close competitor contributes to this range limit (Chapter 2). Many ecological studies have found that trade-offs between abiotic and biotic traits set range limits (Louthan et al. 2015), but very few have investigated potential mechanisms that explain why the traits underlying trade-offs are correlated. This is an important missing link because the mechanism underlying a trade-off dictates how it could be altered/ameliorated (Mauro and Ghalambor 2020). Here, we first proposed a physiological network that could underly a salinity tolerance/competition trade-off by

synthesizing previous physiological work on teleost fish (Fig. 1). We then looked for pathways potentially involved in our trade-off by examining the transcriptome of guppies during a simulated brackish water invasion in the lab. Although there study contains several shortcomings which are addressed, our results provide support that our proposed network may contain pathways important to the trade-off and that these pathways should be investigated further. These results are discussed in detail below.

Our experimental design replicated the field enclosures we previously used in a transplant experiment (Chapter 2) to test how brackish conditions and competing with *P. picta* interacted to reduce *P. reticulata* fitness. We found salinity and interspecific competition decreased body condition (a fitness proxy) in *P. reticulata* (Fig. 2), as observed in the field. The loss in body condition was linked to a change in the social hierarchy of the two species of fish as it was found that *P. picta* become behaviorally dominant over *P. reticulata* in brackish water, but not in freshwater (Fig. 3). This result provides an important link between changes in aggression, competition with *P. picta*, and a loss of fitness in brackish water.

We hypothesized that the observed trade-off and shift in dominance arises in *P. reticulata* because there may be shared biological pathways underlying both aggression and osmoregulation. We then proposed a network that highlights those potentially shared pathways (Fig. 1). To investigate our hypothesis, we used a network analysis to see if the physiological response to salinity influenced aggressive behaviors in *P. reticulata*. Using WGCNA (Langfelder and Horvath 2008), we found that the expression of genes most important to invading brackish water from freshwater (in the presence of competitors) in *P. reticulata* distributed themselves into 13 different modules of co-expressed genes. Of these 13 modules, 4 were significantly correlated with aggression, two associated with a decrease in aggression and two associated with

an increase in aggression, suggesting a potential role for pleiotropy (Table 1). We had identified *a priori* pathways that might result in a trade-off and pathways that could potentially ameliorate the trade-off (Fig. 1; see introduction). Consequentially, we found evidence that both of those types of pathways were embedded in a single module, the turquoise module, which was associated with a decrease in aggression, large, (Table 1) and enriched for GO terms important to osmoregulation (Table 2). We found that a module positively associated with aggression (the green module) was represented in our proposed network as well (Fig. 1) and that it was enriched for cell proliferation, metabolic pathways, and mitochondria function (Table 2). Our results provide evidence that salinity tolerance and aggression may indeed share parts of their physiological pathways, that the pathways we predicted to potentially underscore the trade-off and to potentially ameliorate the trade-off are *both* associated with a decrease in aggression and contained within a *single* module, and that various aspects of energy expenditure are positively linked to aggression. If these results are accurate, alterations to the network shared between salinity tolerance and aggression, specifically within the turquoise module, will be needed to ameliorate the trade-off.

We now ask how amenable to change might this network be? Our study implicates a case of hormonal pleiotropy (McGlothlin and Ketterson 2008) as a possible cause of the trade-off because we used gene expression to uncover a network that is presumably mediated by hormones (i.e. AVT, somatostatins, cortisol, IGF, GH; see Fig. 1). Although genetic pleiotropy and hormonal pleiotropy are often inextricably linked (McGlothlin and Ketterson 2008; Mauro and Ghalambor 2020) differences in their interpretation arise. For instance, changes in binding proteins or receptors can alter the effects hormones have on the suite of traits they effect and may require fewer changes at the genetic level than in a case of genetic pleiotropy (Di Poi et al. 2016;

Bourg et al. 2019). Indeed, small alterations to genes underlying melanocortin receptors have been shown to alter how the hormone MSH affects color without affecting its role in regulating other traits (Rosenblum et al. 2004; Hoekstra et al. 2006; Ducrest et al. 2008; Mauro and Ghalambor 2020). In our system, we speculate that plastic or genetic alterations to implicated receptors/binding proteins like fkpb5, igfpb1a, and igf1rb (Table 2) could have a dramatic effect on the trade-off. Yet, without future manipulative experiments (e.g. CRISPER, pharmacological, etc) we can only speculate because studies on hormonal pleiotropy demonstrate a wide-range of difficulties in which trade-offs arising from hormonal pleiotropy can be altered (Ketterson and Nolan 1999; McGlothlin and Ketterson 2008; Hau and Wingfield 2011). We potentially uncovered one avenue to ameliorate the trade-off: alter energy expenditure, as evident by the pathways within the green module. This is not surprising because it is well understood that osmoregulation and aggression require increases in energy (Gilmour et al. 2005; Tseng and Hwang 2008). However, we argue this not a realistic path to ameliorating the trade-off as energy allocation trade-offs represents intrinsic trade-offs with few solutions (Martin 2015).

Before we conclude, we point out shortcomings of our analysis. We did not control for the fact that the fish in our experiment had not interacted with their competitors before the experiment which means that any variation generated by the interaction between the "shock" of seeing a novel individual and treatment type would be confounded in our analyses. Specifically, the correlations between module expression and aggression could be driven by using "brackish water invasion genes" in our WGCNA that were differentially expressed because of this confounding effect instead of being differentially expressed because of a change in salinity. However, because we interpreted our transcriptomic results in reference to a network formulated *a priori* using previous physiological work, our results may be somewhat robust to this effect.

Further, the turquoise module is functionally enriched for osmoregulatory functions (Table 2) which suggests it contains genes that are indeed important to transitioning between different salinities. Yet, we cannot explicitly account for this potentially confounding effect. This issue in our study relates to a larger shortcoming of many transcriptomic experiments which try to relate lab results to ecological phenomenon in nature. Namely, gene expression is highly plastic and influenced by environmental/treatment conditions which means it is both difficult to account for all potential sources of variation during experiments in the lab and difficult to capture all potential sources of variation that may be present in nature (Gibson 2008). Because of these various shortcomings we suggest our results be viewed as "hypothesis-generating" or "investigatory" rather than providing strong evidence for the pathways that underly this trade-off. As mentioned above, we advocate that future studies directly test the effect of the pathways implicated here on aggression and salinity tolerance by experimentally manipulating them.

Conclusion

One of the great motivators to better understand the adaptive process is so we can better predict the contemporary evolution of populations in response to climate change (Bay et al. 2017). Because climate change is predicted to increase the salinity of coastal rivers and estuaries (Rice et al. 2012; Ghalambor et al. 2021), we predict the trade-off explored here will be problematic for *P. reticulata* inhabiting the coastal rivers of Trinidad. But how generalizable is this trade-off to other fish? We argue that if the biological pathways identified in this study are indeed important to the trade-off, then the trade-off is broadly generalizable to many euryhaline fish because these pathways are conserved osmoregulatory pathways across teleost fish (e.g. Evans et al. 2013). That said, the trade-off appears to lie on a spectrum of expression as evident by *P. picta*. *P. picta*, unlike *P. reticulata*, span the entire salinity gradient in Trinidad, though

inexplicable fail to establish far into freshwater portions of rivers (Torres-Dowdall et al. 2013). Our results show that *P. picta* also experience a change in dominance as they transition between salinities, albeit in the inverse fashion to *P. reticulata*. This suggests that *P. picta* also experience a trade-off between competition and salinity. What is different between the physiological networks of these close competitors that leads to the trade-off manifesting itself in different ways? A comparison of the networks of these two species may offer insights as to how species can alter trade-offs. In all, the work here is one of only a few studies to investigate the mechanism underlying an ecologically relevant trade-off and the first study to formally outline biological pathways that could potentially underly a trade-off between salinity tolerance and aggression in fish.

TABLES AND FIGURES

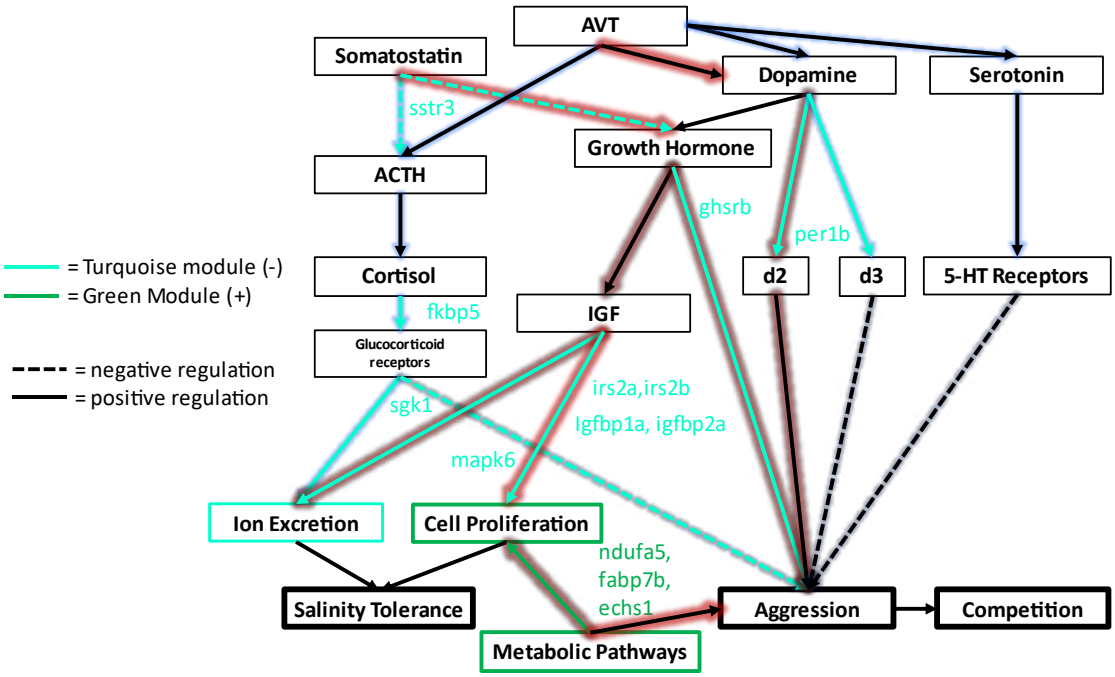

Figure 3.1. A diagram displaying the pathways we identified *a priori* as potentially underlying the trade-off between salinity tolerance and aggression/competition and as potentially ameliorating the trade-off. Biological pathways that were supported based on the results of our functional and network analyses are colored to match the module in which that pathway is found (either the turquoise module which was associated with a decrease in aggression or the green module which was associated with an increase in aggression). Specifically, the pathways colored turquoise or green were supported by integral genes in that colored module (gene symbols are next to the pathways they support) and the colored functions (ion excretion, cell proliferation, metabolic pathways) were supported by enrichment analysis on that colored module. Pathways highlighted in blue represent potential sources of pleiotropy within the network that could underly the trade-off. Pathways highlighted in red represent potential avenues in which the strength of the trade-off could be reduced. Dashed lines represent negative regulation in the direction of the arrow whereas solid lines represent positive regulation in the direction of the arrow.

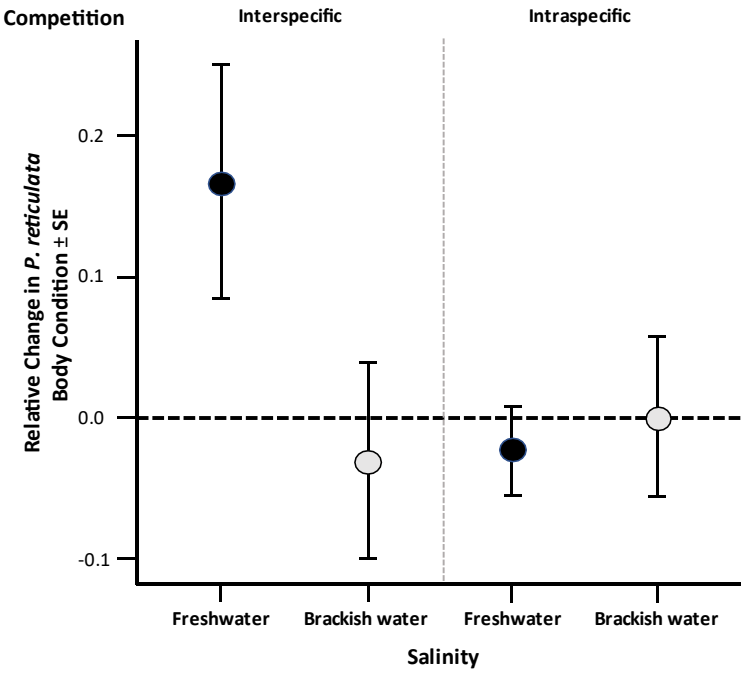

Figure 3.2. The relative change in *P. reticulata* body condition over one week of being under experimental salinity (black circles are treatments in freshwater (0psu) and the grey circles are treatments in brackish water (15psu)) and competitive conditions (left side are interspecific treatments where a *P. reticulata* competed against a *P. picta,* right side are intraspecific treatments where a *P. reticulata* competed against a conspecific). There was no effect of salinity nor competition type, but the interaction between salinity and competition was significant as evident by *P. reticulata* performing significantly better against *P. picta* in freshwater than in brackish water. Means +/- SEs are displayed.

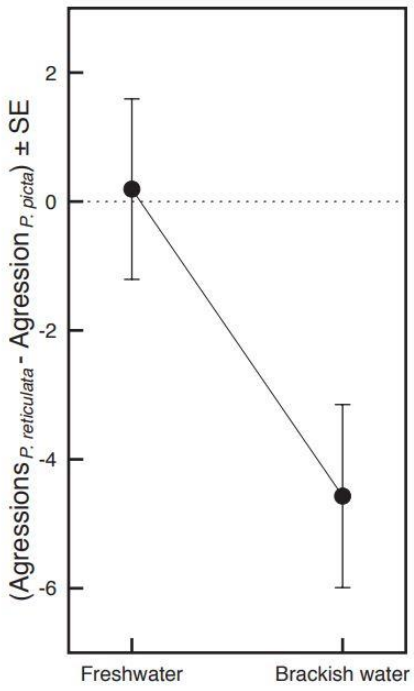

Figure 3.3. The difference in aggressive behaviors between a *P. reticulata* and a *P. picta* in either a freshwater (0psu) or brackish water (15psu) competition tank. Hence, positive values indicate that *P. reticulata* was dominant and negative values indicate that *P. reticulata* was subordinate. *P. reticulata* was neither dominant nor subordinate to *P. picta* in freshwater but was subordinate to *P. picta* in brackish water. Means +/- SEs are displayed.

Table 3.1. A display of key statistics relating to the 13 co-expressed gene modules that were recovered during our WGCNA analysis. Four modules were significantly correlated with aggression and are outlined in black (turquoise and brown modules were negatively correlated and thus associated with a decrease in aggression; green and red modules were positively correlated and thus associated with an increase in aggression). Size of the module, correlation direction, and correlationed p-value are also displayed.

Module	Size (#genes)	Aggression Correlation	Correlation P-value
Tan	33	-0.26	0.2
Brown	155	-0.49	0.02
Turquoise	422	-0.51	0.01
Pink	71	-0.19	0.4
Black	84	-0.065	0.8
Yellow	124	0.02	0.9
Magenta	57	-0.1	0.6
Salmon	31	-0.07	0.7
Green	99	0.58	0.03
Purple	46	0.16	0.5
Blue	164	0.13	0.6
Green-yellow	43	0.32	0.1
Red	92	0.57	0.004
Ungrouped	254	0.29	0.2

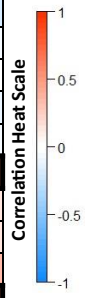

Table 3.2. A table displaying a subset of enriched functions (GO or KEGG) and a subset of integral genes most relevant to the trade-off for the turquoise and green modules. The parentheticals by each term refers to the type of enrichment: MF= Molecular function (GO), BP= biological process (GO), CC= cellular component (GO), KEGG=KEGG.

Module	Enriched Function (type)	Key Genes
Turquoise	• Insulin receptor binding (MF) • Solute: sodium symporter activity (MF) • Enzyme inhibitor activity (MF) • Solute: cation symporter activity (MF) • FoxO signaling pathway (KEGG)	• sgk1 • irs2a, irs2b • mapk6 • irs2a • fkbp5 • Igf1rb, igfbp1a • ghsrb
Green	• Cell proliferation (BP) • Mitochondrial membrane part, protein complex, inner membrane (CC)	• ndufa5 • fabp7b • echs1

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CHAPTER 4:

NEUTRAL AND ADAPTIVE GENETIC DIFFERENTIATION IN A EURYHALINE FISH DISTRIBUTED ACROSS STEEP SALINITY GRADIENTS[4]

SUMMARY

Salinity can have a powerful impact on the abundance, community composition, and distribution of genetic variation in natural populations of euryhaline fish. This is in part because of the osmoregulatory challenge created by changes in salinity, which can lead to divergent selection and reduced gene flow across salinity gradients. For fish that maintain a homeostatic internal osmolality (most euryhaline fish), they must actively secrete ions in hyperosmotic conditions, but do the reverse in hypoosmotic conditions. There is a high physiological cost to switching between these strategies which may cause fish to avoid dispersing between hypoosmotic and hyperosmotic environments. Hence, we hypothesized that the iso-osmotic point, the point between hypoosmotic and hyperosmotic environments, creates population genetic differentiation along salinity gradients. We tested this hypothesis and more broadly examined how salinity influences neutral and adaptive genetic variation in populations of a euryhaline fish, *Poecilia picta*, along repeated steep salinity gradients in an estuary in Trinidad. Specifically, we tested if the iso-osmotic point serves as a barrier to gene flow, if salinity influences population structure while controlling for riverine distance, if there is evidence that salinity drives differentiation at putatively adaptive loci, and if populations at extreme ends of the salinity gradient show signs of local adaptation. We find mixed support for the role of the

[4] Publishing Information: Alexander A. Mauro, Elias M. Oziolor, Brenna R. Forester, Julián Torres-Dowdall, Andrew Whitehead, and Cameron K. Ghalambor

iso-osmotic point contributing to reduced gene flow. However, we find evidence for salinity's ability to drive differentiation at putatively adaptive loci, despite apparently high levels of gene flow. Lastly, we do not find evidence for local adaptation at extreme ends of the salinity gradient when measuring juvenile growth rate. Ultimately, this study adds to growing evidence that salinity has the power to influence genetic variation over short distances in fish in estuaries.

INTRODUCTION

Salinity has a strong influence on the distribution and community composition of fishes, particularly in estuaries where salinity gradients form between oceans and rivers (Martino and Able 2003; Barletta et al. 2005). In some cases, species track seasonal and daily changes in salinity (Barletta et al. 2003; Torres-Dowdall et al. 2013) while in other species salinity acts as barrier to movement and shapes their distributional limits (Whitehead 2010; Torres-Dowdall et al. 2013). Salinity should influence the population structure of fish distributed across salinity gradients if salinity is a driver of divergent selection and local adaptation as that can lead to restricted gene flow between populations along environmental gradients (i.e., isolation by environment; Sexton et al. 2014). Indeed, prior studies have found that salinity influences genetic differentiation in fish populations occurring across salinity gradients (McCairns and Bernatchez 2008; Whitehead et al. 2011; Berg et al. 2015; but see Regmi et al. 2016). In particular, there is growing evidence that populations exhibit a bipartite division along continuous salinity gradients in which a freshwater ecotype and a brackish water ecotype emerge despite no barrier to dispersal between the groups and, in some cases, in the face of ongoing gene flow (McCairns and Bernatchez 2008; Whitehead et al. 2011; Dennenmoser et al. 2017).

Salinity influences population genetic structure in fish at least in part because of the differential osmoregulatory challenge it poses (Evans et al. 2013). Most euryhaline fish maintain

a homeostatic internal osmolality (an iso-osmotic point) of 12psu (Evans et al. 2013). Hence, euryhaline fish have mechanisms that allow them to osmoregulate i.e. expel salt ions and increase water retention in hyperosmotic conditions (conditions above the 12 psu iso-osmotic point) and do the reverse in hypoosmotic conditions (conditions below the 12 psu iso-osmotic point) (Evans et al. 2013; Kultz 2015). Thus, any population that spans salinity levels above and below the iso-osmotic point should be subject to strong divergent selection (Whitehead et al. 2011; Schultz and McCormick 2012). However, osmoregulatory ability is a highly complex physiological trait that involves many different organs (brain, kidney, intestine, gills) (Laiz-Carrión et al. 2004; Evans et al. 2005; Arjona et al. 2007; Edwards and Marshall 2012) and coordinated changes across different levels of biological organization (McCormick 2001; Evans and Somero 2008; Evans et al. 2013; Norman et al. 2014). As such, numerous candidate genes associated with osmoregulatory pathways have been identified as being under selection and leading to local adaptation to different salinity levels (e.g. Kozak et al. 2014; Guo et al. 2015; Dennenmoser et al. 2017). However, osmoregulation is also a highly plastic trait in euryhaline fish and it is well understood that salinity induced changes in gene expression within the gills facilitate ion exchange (Evans et al. 2005; Hwang et al. 2011). The gills exchange ions through specialized mitochondrion-rich cells called ioncytes that exchange ions via ion-transporters (Hwang et al. 2011; Edwards and Marshall 2012). Under hyperosmotic conditions ionocytes are proliferated and under hypoosmotic conditions ioncytes are removed. These plastic changes are largely endocrine controlled by insulin-like growth factors and cortisol (Sakamoto and Hirano 1993; Björnsson et al. 2002; Mancera and Mccormick 2007). Thus, local adaptation might result from selection on signaling molecules, receptors, transporters and other genes that influence the *capacity* for adaptive plasticity to salinity challenges (e.g. Norman et al. 2011; Kozak et al.

2014; Kusakabe et al. 2017) and this may be a common mechanism in which populations locally adapt to different salinity environments (Schulte 2007; Whitehead 2010; Divino et al. 2016).

Here we examine population genetic variation in a euryhaline fish species, *Poecilia picta,* on the island of Trinidad, where populations are repeatedly distributed across a steep salinity gradient (Torres-Dowdall et al. 2013). We first investigate how this pattern might be reflected in the genomes (using low-coverage whole genome sequencing) of populations of *P. picta* along salinity gradients in three different rivers (Fig. 1). We test the hypothesis that salinity can restrict gene flow and create population structure. Specifically, we test if population structure is best explained by treating salinity level as a continuous impediment to gene flow (isolation by environment, IBE) or by treating the iso-osmotic point in a river as a distinct barrier to gene flow (isolation by adaptation, IBB) while controlling for riverine distance between populations (isolation by distance, IBD), relative to the null hypotheses of panmixia and isolation by distance (Fig. 2A).We next test the hypothesis that salinity can drive divergent selection at putatively adaptive loci between populations along the salinity gradient despite high levels of gene flow. Lastly, we test for evidence of local adaptation to salinity by measuring juvenile growth rate of a freshwater population and a high salinity population to test if any putative adaptive genomic differences are reflected at the phenotypic level.

MATERIALS & METHODS

Fish Collection and Sample Preparation

Adult *P. picta* individuals were caught at 11 sites (range 20-50 individuals per site) along a salinity gradient in the Caroni, Caparo, and Cunupia rivers in Trinidad during March 2017 (Fig. 1). Within each river, *P. picta* were caught at a 1) freshwater site (0psu), 2) low salinity site (1-5psu), 3) medium salinity site (16psu, population missing from the Cunupia river), and 4) high

salinity site (20-23psu) (see Table S38 for latitude/longitude coordinates for each sampling site). Fish were transported live back to Hamgel Field Station in Trinidad, sacrificed via decapitation followed by a brain pith, and tail fin clips were taken and preserved in 90% ethanol at -20˚C. Fin clips were then transported back to a molecular laboratory for processing at Colorado State University. Genomic DNA was extracted from tail fin tissue using a QIAGEN DNeasy protocol with an optional RNase treatment included. Library preparation was conducted at the University of California Davis by shearing extracted DNA into ~500bp fragments using a Covaris220, and genomic libraries were prepared using NEBNext Ultra DNA library kits with unique individual indexes following the protocol described in Oziolor et al. (2019).

Sequencing, Alignment, and Variant Calling

Genomes of 350 *P. picta* were sequenced at Novogene Sequencing Center via PE150bp sequencing on Illumina HiSeq X10. We used FastQC (Andrews 2010) to assess quality and cutadapt (Martin 2011) to remove adapters and reads below a phred score of 30 and/or reads shorter than 36bp. Reads were mapped to the *Poecilia reticulata* genome (Kunstner et al. 2016) using bowtie2 v 2.02 (Langmead and Salzberg 2012). Samtools (Li et al. 2009) was used to assess coverage, and sort and index reads. Each sample had roughly 9.7 million reads and 2x coverage across the genome (~731Mb genome). We excluded bases with abnormally high coverage (>200x) (roughly 14Mb or 1.6% of the genome). We used Freebayes (Garrison and Marth 2012) to call variants and vcftools (Danecek et al. 2011) to filter our variant calls to a set of bi-allelic SNPs with a minimum allele frequency of 0.01, minimum quality score of 30, and with at least 50% of samples having the SNP. This yielded 19.8 million biallelic variant sites for further analyses.

Population Statistics, Structure, and Ancestry

We used ANGSD (Korneliussen et al. 2014) to estimate a summary statistic for genetic diversity, π. To do this, we first estimated allele frequency spectra using 50mb of the genome from each individual. Sites were filtered in the same fashion as described above. These estimated frequency spectra were then used as priors in a Bayesian procedure in ANGSD to estimate per site values across the genome. Per site estimates were combined into sliding windows of 50kb, moving in 10kb increments.

To assess population structure, we calculated genome-wide pairwise F_{ST} values. We used VCFLIB (Cleary et al. 2015) to estimate all pairwise F_{ST} values from called genotype likelihoods using Weir and Cockerham's theta (Weir and Cockerham 1984). This approach has been to shown to be a powerful and robust way to calculate F_{ST} across entire genomes (Kitada et al. 2007). We used ADMIXTURE (Alexander et al. 2009) to estimate ancestry. To do this, we fist filtered out variants in linkage disequilibrium due to physical linkage in the genome via the methods recommended by Dutheil (2020) and then estimated values for the number of ancestral populations "k" for all 11 sites (k=1-6 evaluated). To select a k value, we used the cross-validation procedure built into ADMIXTURE (10-fold CV) (lower values indicating a better value of k).

Determination of Outlier Genes and Functional Enrichment of Outlier Genes

We conducted three different outlier tests to identify regions potentially associated with adaptation to different levels of salinity. First, we conducted a F_{ST} based outlier test in which we identified loci highly differentiated between freshwater populations and high salinity populations. Specifically, we used the population branch statistic (PBS) from Yi et al. (2010). PBS is an effective way to identify outlier loci if one has an *a priori* idea of a potential driver of selection (salinity in our case) and has been effective in identifying outlier loci in other aquatic

systems (Reid et al. 2016; Oziolor et al. 2019). It allows for the comparison of a target population, in a group of interest, against the background differentiation between two populations in a comparison group. Hence, we compared each freshwater population against the high salinity population in its river and the next nearest high salinity population (here on referred to as the PBS test). We then took the top 1% outlier loci from each test and looked for associated genes. To do that, we used the intersect function in BEDTools (Quinlan 2014) to identify mapped genes that intersected with our outlier regions.

In our second outlier test, we attempted to target loci that showed characteristic signs of a selective sweep by identifying loci that were both highly differentiated (as measured by F_{ST}) and had low levels of genetic diversity (low π) (Cutter and Payseur 2013). Following Reid et al. (2016) we created a merged-z statistic in which a high z-value would indicate loci that were both highly differentiated and containing low levels of diversity (here on referred to as the z-test). We then conducted a PBS test in the same fashion as described above using the merged-z statistic, took the top 1% outlier loci from each test, and identified genes associated with the outlier loci (same process as above).

Our third outlier test was a genotype environment association test (GEA) using redundancy analysis (RDA). RDA is a powerful GEA method, with low false positive rates when population structure is weak (which is what we have in this system, see admixture results in Fig. 3) (Forester et al. 2018). To perform the RDA, we first used plink2 (Chen et al. 2019) to transform our SNPs into population level allele frequencies for all 11 populations. Then, using the package "vegan" (Oksanen et al. 2020) in R (Team 2019) we conducted an RDA in which salinity was the explanatory variable, quantified as a vector of standardized psu values measured at each capture site. Following the procedure outlined by Forester from an online collection of

population genetic tutorials (referenced as Kamvar et al. 2017) we then took all SNPs that had loading scores on our single RDA axis that were 5 standard deviations away from the average loading score (representing a two-tailed p-value of 0.0000006) and identified genes associated with those SNPs (same process as other two tests).

Between our three types of tests, seven lists of genes were created. To narrow these lists to the most significant and well-supported detections relevant to salinity tolerance, we looked for genes identified from multiple tests and genes known to be associated with our trait of interest, osmoregulation, as has been done other studies utilizing multiple outlier tests (i.e. Dalongeville et al. 2018). Further, we ran functional enrichment tests on our gene lists using ShinyGO (Ge et al. 2020) (with a corrected FDR significance cut-off of 0.05) to better understand the biological qualities of our lists of genes.

Testing alternative hypotheses for the causes of genetic divergence

To evaluate the roles riverine distance (isolation by distance, IBD), salinity (isolation by environment, IBE), and the iso-osmotic point (isolation by barrier, IBB) play in shaping genetic distances in our populations of *P. picta* (Fig. 2A), we used partial RDAs to partition the variation explained by each factor (Lasky et al. 2012). Further, we ran partial RDAs on SNPs from the entire genome, from regions only containing outlier loci (as determined by PBS and z-tests; see above), and from regions only containing neutral loci (the entire genome excluding the outlier regions) to better understand the drivers of genetic differentiation across different parts of the genome (e.g. Orsini et al. 2013). Specifically, we ran partial RDAs on all SNPs, 50k random outlier loci SNPs, and 50k random neutral SNPs. To do this, we again used plink2 (Chen et al. 2019) to transform our SNPs into population level allele frequencies for all 11 populations and

used the "vegan" package (Oksanen et al. 2020) in R (Team 2019) to run partial RDAs. Specifically, we ran an RDA including all factors (IBD/IBE/IBB) as explanatory variables and a series of three partial RDAs in which one of the three factors was used as an explanatory variable while the other two variables were conditioned/controlled (Borcard et al. 2011). Salinity (IBE) was quantified as a vector of standardized psu values measured at each capture site. The iso-osmotic point factor (IBB) was quantified as a vector indicating if each population's capture site was in hyperosmotic conditions or hyperosmotic conditions. We also tried quantifying IBB as a vector describing each population's "salinity" distance from the iso-osmotic (salinity of capture site minus 12psu), but a variance inflation factors analysis revealed this approach led to high collinearity between our factors. Riverine distance (IBD) was more challenging to quantify. In terrestrial systems, a matrix of latitude and longitude has typically been used to model distance between populations, but this is clearly unsuitable to river systems where aquatic organisms can only disperse along waterways (Mozzaquattro et al. 2020). We tried two different options to address this problem. We calculated the riverine distance from each site to the ocean (distance to ocean, DTO) and we calculated the total riverine distance between a site and all other sites (this metric is called "fareness" in network statistics). A variance inflation factors analysis revealed that DTO was the better distance metric to use. However, we could not ultimately avoid collinearity between our three variables.

Growth Rate Under Different Salinities

To test for evidence of local adaptation to different ends of the salinity gradient, we conducted a common garden experiment to test how salinity affected juvenile growth rate in a population of *P. picta* from a high salinity site and from a population from a freshwater site. Specifically, we measured juvenile growth rate of both populations in the freshwater "home"

environment (0psu) and in the high salinity "home" environment (20psu). The full details of the common garden, husbandry of the fish, and phenotyping can be found in Chapter 2 but will be briefly summarized here. Twenty gravid female *P. picta* were caught from a freshwater site (0psu) and from a high salinity site (~20psu) along the Caroni river, transported to Colorado State University, and bred in the lab in recirculating tanks. All experiments were done on F2 generation fish in a split sibling fashion, so family lines (n=12-15 per population) were represented in both salinity treatments. We compared differences in growth rate using a mixed model ANOVA using "lme4" (Douglas et al. 2015) in R in which salinity and population and their interaction were fixed effects and family line was a random effect.

RESULTS

Population Structure & Ancestry Estimates

In general, F_{ST} estimates between populations were low with the average F_{ST} between all populations being 0.026 (Table S1). There was a pattern in the Caroni and Cunupia rivers in which F_{ST} was high between populations on either side of the iso-osmotic point (Fig 2). However, this pattern was not consistent along the full extent of the rivers as F_{ST} was sometimes lower between freshwater populations and high salinity populations than between low salinity and medium salinity populations within the same river (Table S1). Further, F_{ST} values did not consistently reflect proposed riverine distances between populations (Fig 2).

Our admixture results for all 11 populations favors a k of 2 slightly above a k of 1 (Table S2) with populations of the same salinity resembling one another more than populations of the same river (Fig 3). Further, there appears to be a "freshwater" genotype (green bars, Fig 3) that is increasingly more prevalent in populations with lower salinities.

Outlier Genes

To identify genes potentially associated with adaptation to different salinities, we conducted three types of outlier tests: a PBS test based on identifying F_{ST} outliers (see Fig. S2 for a Manhattan plot), a z-test based on identifying areas of the genome that were highly differentiated (F_{ST} outliers) and had low levels of π (see Fig. S1 for population level π results; see Fig. S2 for a Manhattan plot), and a GEA using an RDA (see Table S37 for RDA results). A total of 988 unique genes were identified via the PBS test, 614 unique genes were identified via the z-test, 556 unique genes were identified via the GEA test, and 26 genes were shared between all tests (Fig. 4). Overall, the PBS test and z-test reported many of the same genes whereas the GEA test had low overlap with the other two tests (Fig. 4). This is to be expected as both the PBS test and z-test relied in part on F_{ST} comparisons between the same sets of populations whereas the GEA test identified loci with allele frequencies related to salinity gradients. Still, all three tests picked up on genes and GO/KEGG pathways with known osmoregulatory function (Table 1). Additionally, functional enrichment analyses on the PBS-test and z-test revealed significant enrichment for GO/KEGG categories associated with immune function; however, immune system enrichment was not found for the genes from the GEA (Table 1). Full lists of the genes identified and the full results from the enrichment tests can be found in Tables S3-S33.

Testing IBD vs. IBE vs. IBB

None of the examined RDA models in our partial RDA analysis were significant for any portion of the genome (whole genome, neutral loci, outlier loci) (Table 2; Tables S34-S35). This may have been due to collinearity between our different explanatory variables (VIF: IBD=2.622, IBE=25.15, IBB=19.52). This collinearity is evident in our RDA tables as the variation confounded by salinity/iso-osmotic/DTO is negative.

Growth Rate Under Different Salinities

Developing under high salinity conditions significantly decreased growth rate for both populations of *P. picta* (P=0.002) (Fig 5; Table S36). Furthermore, populations did not differ in their response to salinity (population x salinity interaction; P=0.99) and there was no population effect on growth rate (population P=0.065).

DISCUSSION

Salinity gradients represent unique environmental gradients that are expected to influence patterns of gene flow and selection, but the different ways in which salinity impacts the distribution of neutral and adaptive genetic variation in natural populations of euryhaline fish remains unresolved. For example, studies have found putatively adaptive loci associated with osmoregulation differentiated between freshwater and saltwater populations of euryhaline fish despite gene flow between populations (Kozak et al. 2014; Berg et al. 2015; Dennenmoser et al. 2017). However, the different mechanisms by which salinity creates this pattern through altered gene flow and divergent selection have not been explicitly tested. One hypothesis that reflects the physiology of euryhaline fish is that the iso-osmotic point serves as a barrier to gene flow because of the osmoregulatory cost of switching between hypoosmotic and hyperosmotic environments. We tested this hypothesis and other alternatives for how salinity influences the distribution of population genetic variation in a euryhaline fish by examining whole genome sequences of 11 different populations of *P. picta* from three different Trinidadian rivers. We found evidence for high levels of population connectivity and gene flow within rivers and between rivers (Fig. 2; Table S1) and mixed evidence that the iso-osmotic point acts as a barrier to movement (Fig. 2). However, despite high levels of gene flow we found evidence for salinity as a source of divergent selection on loci associated with osmoregulation and immune function (Table 1). These results are discussed in more detail below.

Neutral genetic variation

Overall, genome-wide genetic differentiation between all populations, regardless of river, in our study system was low (average pairwise F_{ST} = 0.026, Table S1). This was somewhat surprising as there are no waterways that link our study rivers except via the ocean (Fig. 1). The lack of genetic differentiation and presumably high levels of gene flow among rivers (Fig. 2) suggests two possible ways in which populations are connected. First, there may be substantial movement of individuals along the coastline, particularly during the wet season when high flows could push individuals into the ocean and allow them to colonize other river systems. Second, our sampling sites may be readily connected via temporary streams during flooding events in the wet season. We sampled during the dry season, so temporary connections between rivers within the larger estuary that they inhabit (Fig. 1) would not have been apparent. This lack of differentiation between populations was further reflected in our ancestry estimates as our ADMIXTURE results support a k of 2 (Fig. 3). Formally testing the hypothesis that dispersal through the ocean or temporary flooding connects populations is possible (i.e. Karim et al. 2012), but requires additional hydrological and/or genetic data beyond the scope of this study.

Despite the general lack of population structure and high population connectivity, we identified qualitative support for a bipartite division along the salinity gradient via the iso-osmotic point hypothesis i.e., a division between a freshwater/low salinity group and a medium/high salinity group. Our genome-wide ADMIXTURE results support this hypothesis as the two clades favored reflect the split between low and high salinity levels (Fig. 3). Further, some of the highest F_{ST} values between populations were between populations on either side of the iso-osmotic point (Fig. 2). However, some of the lowest F_{ST} values were between populations at the extreme ends of salinity gradients (Table S1) suggesting barriers to gene flow are not due

to the iso-osmotic point alone. We explored these ideas further by examining the influence of salinity as a continuous environmental gradient, the influence of the iso-osmotic point, and the influence of riverine distance on population structure using partial RDAs. However, none of the RDA models examined were significant (Table 2; Tables S34-S35). The lack of significance could reflect the collinearity between explanatory variables (specifically between salinity and iso-osmotic variables). It is possible that future analyses could overcome this by redefining variables using more ecologically relevant approaches. Further, although riverine distance was not collinear with other variables, it could be redefined using distance based Moran's eigenvector maps (dbMEMs) (Dray et al. 2006; Borcard et al. 2011) or using a graph-theory approach designed for dendritic topologies like rivers (Urban and Keitt 2001; Mozzaquattro et al. 2020) which could also improve the model.

Loci under selection

Despite a lack of genome wide population differentiation, we found evidence for salinity as a source of divergent selection on specific loci. We conducted three different outlier tests: one aimed at finding outlier loci between freshwater populations and high salinity populations (PBS-test), one aimed at finding genomic regions showing signs of selective sweeps (z-test), and a GEA using salinity as an explanatory variable. All three tests uncovered outlier loci associated with known osmoregulatory genes important to ion exchange in the gills either via ion transporters or the regulation of ionocytes (Table 1). Specifically, we identified sodium ion transporter genes in all three tests and the gene lists from all tests were enriched for ion transport (Table 1). Further, all three tests found genes associated with insulin growth factor which is known to regulate ionocytes in the gills and the genes from all tests were enriched for functions known to control cell proliferation during osmoregulation (MAPK signaling pathway, apoptosis)

(Sakamoto and McCormick 2006; Edwards and Marshall 2012; Kültz 2015). Collectively, these genes reflect the pathways associated with the plastic response to salinity (Reid S Brennan et al. 2015; Kultz 2015; Lema 2020) and suggest divergent selection may not be acting on constitutive differences in osmoregulatory ability, but rather the speed and magnitude of mounting an osmoregulatory response to fluctuating salinity. Indeed, populations of the euryhaline Atlantic killifish (*Fundulus heterclitus*) occupying freshwater and brackish water exhibit divergence in genes involved in acute and acclimatory responses to salinity challenge (Whitehead et al. 2011; Reid S Brennan et al. 2015). Thus, local adaptation to different salinity levels in euryhaline fish appears to reflect divergence in the speed and magnitude of adaptive plasticity to osmotic challenges (Whitehead et al 2011; Brennan et al. 2015).

We also found evidence for divergent selection on loci associated with immune functions. Both the z-test and PBS-test identified genes enriched for immune function, most notably the MHC protein complex (Table 1). Immune function genes, and particularly MHC genes, commonly exhibit patterns of selection in genomic outlier tests in fish (Hansen et al. 2007; Jensen et al. 2008; Tonteri et al. 2010). Several hypotheses could explain divergence in *P. picta* immune functions across the salinity gradient. First, parasite load in *P. picta* and parasite community are known to differ across salinities in Trinidad (Robison 2018), hence parasites could be one driver in the differentiation of MHC genes. Second, osmoregulation encompasses several overlapping biological pathways (Barton 2002; Kultz 2015), such that there may be pleiotropic connections between immune function and osmoregulatory ability (Cuesta et al. 2007). Indeed, cortisol and growth hormone both play critical roles in osmoregulation (McCormick 2001; McCormick and Bradshaw 2006; Sakamoto and McCormick 2006) and are known to directly impact immune function in fish (Harris and Bird 2000; Barton 2002; Yada

2007; Tort 2011). For example, transgenic zebrafish (*Danio rerio*) that overexpress growth hormone have impaired immune systems (Batista et al. 2014), suggesting a role for correlated responses to selection between the endocrine, osmoregulatory, and immune systems.

No evidence for local adaptation in growth rate

The identification of putatively adaptive loci via genome scan methods should ultimately be viewed as hypothesis-generating tests with regards to the evidence they provide for local adaptation (Barrett and Hoekstra 2011). Phenotypic comparisons of traits associated with the outlier loci between populations, ideally in a common garden (or reciprocal transplant) experiment, should be conducted to directly investigate local adaptation between populations (Kawecki and Ebert 2004; Savolainen et al. 2013; Defaveri and Merilä 2014). We took this approach to test for local adaptation at the extreme ends of our salinity gradient. We found that a freshwater population and a high salinity population in the Caroni river did not differ in terms of their juvenile growth rate in two different salinities reflecting their respective home environments (Fig. 5). Hence, we did not find evidence for local adaptation in this trait. We also found that both populations grew better in freshwater (Fig. 5). This pattern has been found in other studies that measured juvenile growth rate in populations of fish along a salinity gradient (Defaveri and Merilä 2014). However, in light of the outlier loci we found and the complimentary work suggesting that local adaptation in euryhaline fish may reflect changes in the plastic responses to salinity challenge, measuring constitutive differences in growth rate was perhaps not the best test. Comparisons of the time course of transcriptomic responses or the speed at which gills are remodeled in response to salinity challenge (i.e. Whitehead et al. 2011; Brennan et al. 2015) may provide more robust tests of local adaptation.

Conclusion

We found evidence that salinity can drive differentiation at putatively adaptive loci despite high population connectivity in our estuarine system. These results add to a growing list of studies that find evidence that salinity can drive putatively adaptive loci to differentiation even at geographically small spatial scales (Conover et al. 2006; Defaveri et al. 2011; Shimada et al. 2011; Guo et al. 2015). However, how divergence at these putatively adaptive loci translates into patterns of local adaptation to different salinity levels is complicated by the highly polygenic and plastic nature of osmoregulatory responses. Given their wide distribution and general lack of population genetic structure, it appears *P. picta* is generally tolerant of a wide range of salinity levels, but populations at the opposite ends of the salinity gradient differ in the degree to which they cope with fluctuating salinity levels through plasticity.

FIGURES & TABLES

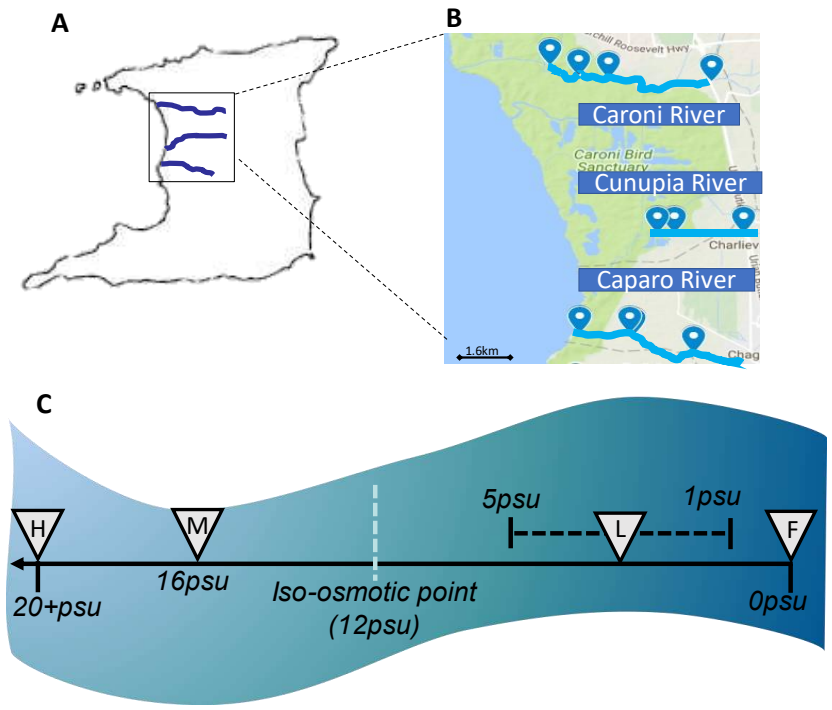

Figure 4.1. A-B) We sampled *P. picta* from 11 different sites across 3 rivers along the western coast of Trinidad: the Caroni, Cunupia, and Caparo rivers. C) We sampled in such a way to capture the entire salinity gradient inhabited by *P. picta*. We sampled individuals from a freshwater site (0psu), low salinity site (1-5psu), medium salinity site (16psu), and high salinity site (20+psu) along all 3 rivers (note we are missing a medium salinity site in the Cunupia river). The iso-osmotic point (12psu), the point at which the external salinity would match the internal salinity of *P. picta*, is shown as a reference point.

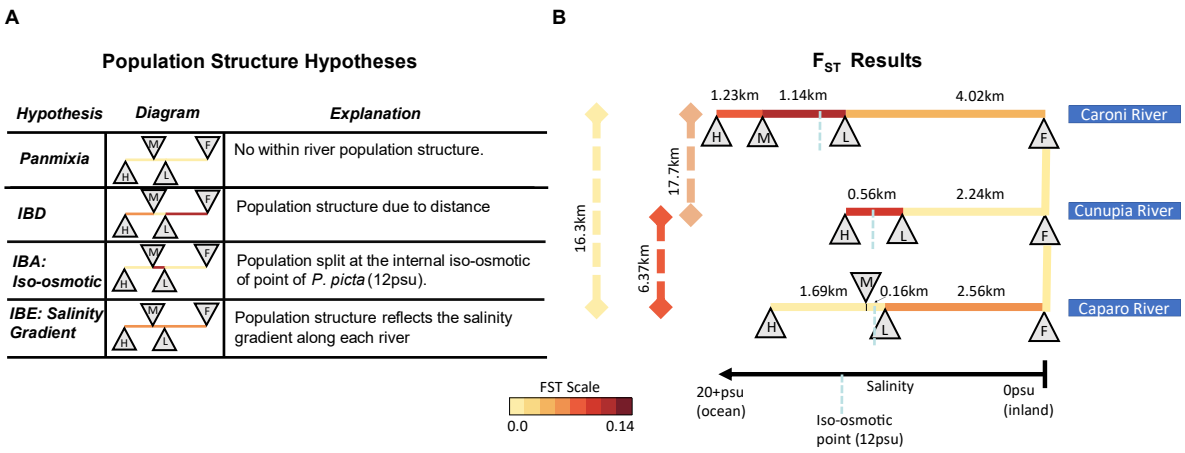

Figure 4.2. A) A diagram outlining 4 non-mutually exclusive hypotheses that we investigated regarding what factors might influence genetic distances between our sampled populations. Specifically, we investigated if there was no population structure (panmixia), if genetic distance was influenced by riverine distance (IBD), if genetic distance was influenced by being above or below the iso-osmotic point (IBB), and if genetic distance was influenced by the salinity of the capture site (IBE). B) A diagram that puts our F_{ST} results in context of the genetic hypotheses investigated by mapping pairwise F_{ST} values, iso-omotic points, pairwise riverine distances, and salinity of capture sites on the same map (note this map is only drawn to a rough scale). These hypotheses were further investigated by the results found in Figures 3 and Table 2.

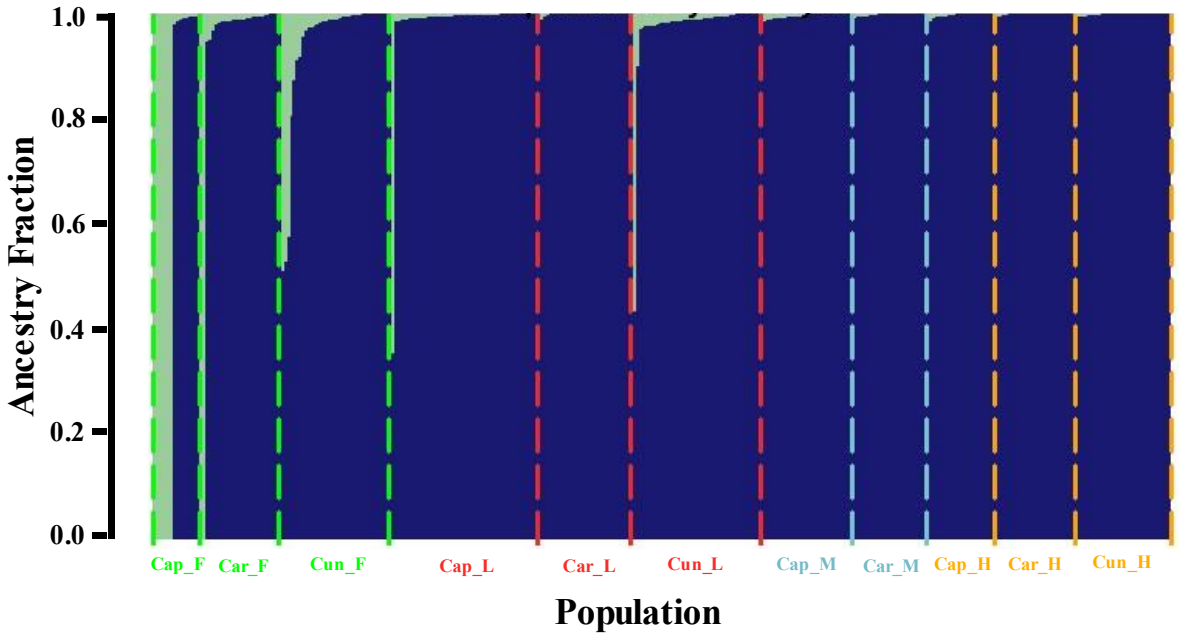

Figure 4.3. A plot showing ancestry fractions for every individual in our 11 populations for an ancestral population value (k) of 2 (most favored value from our ADMIXTURE results, see Table S2). Individuals are represented by a bar and are organized by sampling salinity (freshwater (F), low salinity (L), medium salinity (M), and high salinity (H)) and river (Cap=Caparo, Car=Caroni, Cun=Cunupia). Most individuals are descendent from a "blue" ancestor population, but as salinity decreases an increasing number of individuals have portions of their genome descendent from a "green" ancestor population.

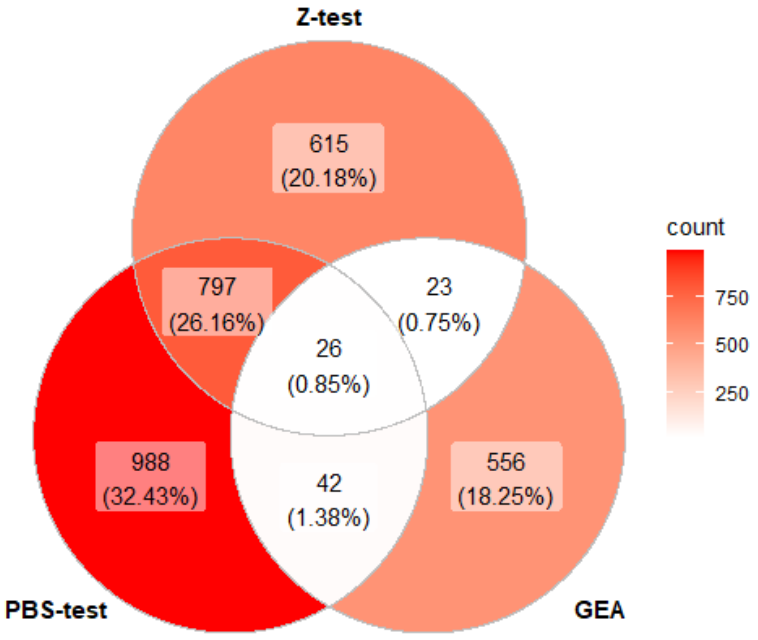

Figure 4.4. A Venn diagram documenting the unique and shared genes between all types of outlier gene tests conducted (PBS-test, z-test, GEA; see methods for details). Overall, many unique genes were found by all three tests, a fair number of genes were shared between the PBS-test and z-test, and few genes were shared between all three tests.

Table 4.1. Key genes and significant functional enrichment categories (MF=molecular function (GO), CC=cellular component (GO), KEGG=KEGG) from all outlier tests with a record of whether each gene/category was found in each test. Categories/genes are further broken down by their association with osmoregulation and/or the immune system (far left bars).

		Z-Test			PBS Test			GEA	
	GO/KEGG/Gene	Caparo	Caroni	Cunupia	Caparo	Caroni	Cunupia	All Pops	Total
Osmoregulation	Insulin growth factor genes (irs2a/igfbp2b) or Insulin signaling pathway (KEGG)		X	X	X	X	X	X	6
Osmoregulation	Sodium transporter genes (slc12a3, slc13a1, slc24a5, slc9a9, slc8a2b)		X	X		X	X	X	5
Osmoregulation	Anion:cation symporter activity (MF)/Cation:cation antiporter activity (MF)			X			X	X	3
Osmoregulation	MAPK signaling pathway (KEGG)			X				X	2
Osmoregulation / Immune System	Apoptosis (KEGG)	X		X		X	X		4
Immune System	MHC protein complex (CC)	X	X				X		4
Immune System	Intestinal immune network for IgA production KEGG)	X				X	X		3

Table 4.2. Full results from our partial RDA analysis from SNPs from the entire genome (results from only neutral loci and only outlier loci are essentially identical to the results here, see Tables S34-35 for those results). No model was significant and due to some collinearity between explanatory factors confounded inertia was negative.

Name	Model	Constrained Inertia	Adjusted R2	P-value - from ANOVA	Proportion of explainable variance	Proportion of total variance (constrained/total)
Full model	F ~ RD + salinity + IO	102730.00	0.060	0.29	1.00	0.34
Pure river distance (RD)	F ~ RD + | (salinity + IO)	36000.00	0.030	0.28	0.35	0.12
Pure salinity	F ~ salinity | (RD + IO)	32830.00	0.020	0.25	0.32	0.11
Pure iso-osmotic (IO)	F ~ IO | (RD + salinity)	44040.00	0.070	0.23	0.43	0.15
Confounded	RD/salinity/IO	-10140.00			-0.10	
Total unexplained	from full model	197170.00				
Total inertia	from full model	299900.00				

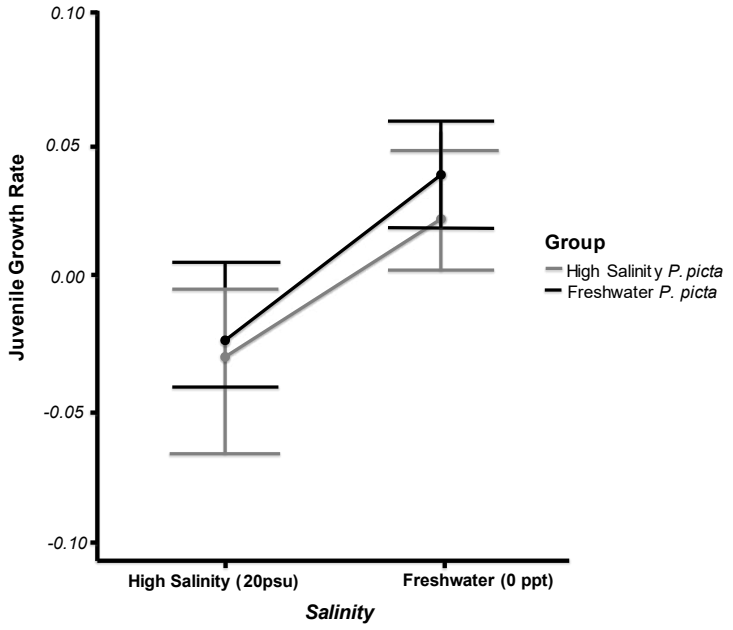

Figure 4.5. Juvenile growth rate under freshwater and high salinity conditions in F2 *P. picta* from a freshwater population (black) and from a high salinity population (grey). Both populations grew faster in freshwater and responded similarly to salinity.

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APPENDICES

Chapter 2

Table. S2.1. ANOVA table for the effect of salinity and food level on juvenile growth rate in full-sibling G2 *P. reticulata* family lines. Salinity and food level and their interaction were treated as fixed factors and family line was treated as a random variable.

Factor	Sum Sq	Mean Sq	NumDf	DenDF	F-value	P-value
Salinity	24.560	24.560	1	122.45	36.8004	1.504e-08
Food	157.741	78.871	2	116.55	118.1617	2.2e-16
Salinity:Food	6.045	3.023	2	115.32	4.5287	0.01278

Table. S2.2. Least-squared means contrasts for the model from Table S1. At each food level, brackish (B) and freshwater (F) groups were compared with a Tukey adjustment for multiple comparisons.

Contrast	Food Level	Contrast Estimate	SE	df	T-ratio	P-value
B-F	High	-0.384	0.230	121	-1.671	0.0972
B-F	Medium	-0.912	0.225	118	-4.050	0.0001
B-f	Low	-1.643	0.360	118	-4.571	<0.0001

Table. S2.3. ANOVA table for effect of two treatments, brackish water in the absence of competitors or freshwater in the presence of a *P. picta* competitor, on the juvenile growth in full-sibling G2 *P. reticulata* family lines. Treatment was treated as a fixed factor and family line was treated as a random factor (family: variance=2.70017, sd=1.6437; residual: variance=0.6451, sd=0.8032)

Factor	Sum Sq	Mean Sq	NumDf	DenDF	F-value	P-value
Treatment	17.796	17.796	1	9.8655	27.588	0.0004

Table. S2.4. The results of a Spearman rank correlation test for the ten family lines from Table S3.

Test Statistic	Rho	P-value
Value	-0.6364	0.05445

Table. S2.5. ANOVA table for the effect of salinity and competition type on survival of *P. reticulata* in field enclosures. Salinity and competition type and their interaction were treated as fixed factors and natural log transformed mass was treated as a covariate.

Factor	Chisquare	Df	P-value
Salinity	8.2285	1	0.004124

Competition	2.2412	1	0.134379
Salinity:Comp	9.5736	1	0.001974
Ln_mass	1.9872	1	0.001974

Table. S2.6. Least-squared means contrasts for the model from Table S5 in which comparisons between each salinity are made for each level of competition (the interspecific competition treatment being the 25% conspecific competition level and the intraspecific competition treatment being the 100% competition level). Statistics reflect a Tukey adjustment for multiple comparisons.

Salinity Contrast	Competition Level	Contrast Estimate	SE	df	Z-ratio	P-value
F-S	25% conspecific	2.926	1.219	inf	2.401	0.0164
F-S	100% conspecific	-0.479	0.417	inf	-1.148	0.2508

Table.S2.7. ANOVA table for the effect of salinity on behavioral dominance. Salinity was treated as a fixed factor and fish ID as a random factor.

Factor	Sum Sq	Mean Sq	NumDf	DenDF	F-value	P-value
Salinity	335.98	335.98	1	13.986	5.7123	0.03148

Ethogram

1. **Nip**: "An individual bites or attempts to bite its opponent. The nipped fish often makes a rapid retreat." (Magurran & Seghers 1991)
2. **Rapid Approach or Chase** (RA/Chase): "An individual swims rapidly towards an opponent, usually aiming at its flank and may attempt to nip it. The attacked guppy usually responds by fleeing" (Magurran & Seghers 1991)
3. **Guarding or Patch Monopoly** (PM): "Individual actively defends patch and chases/herds all other foragers away" (Magurran & Seghers 1991).

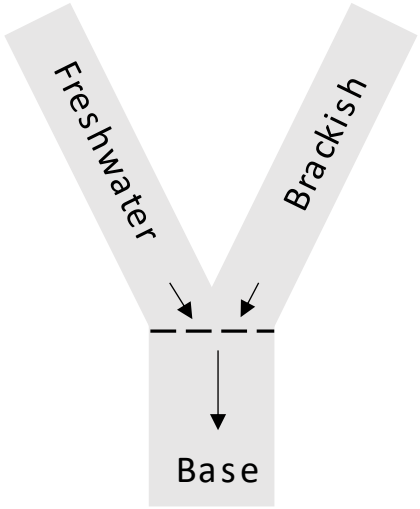

Figure S2.1. A schematic of the y-maze choice experiment. Fish were held in the base arm for a 10-minute acclimation period. Then freshwater and brackish water were simultaneously released from the two response arms and a continuous flow into the base arm was maintained throughout the experiment. Each fish's preference between the two response arms was recorded (we considered a preference to have been made when a fish left the base arm and stayed in the selected arm for at least two minutes).

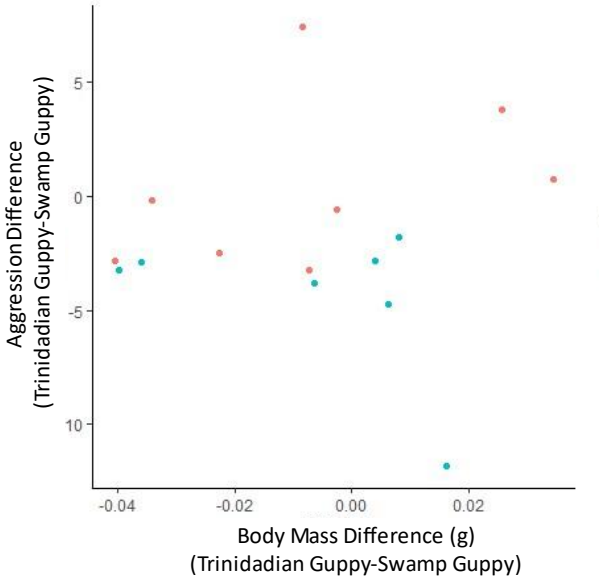

Figure S2.2. A plot showing the relationship between differences in aggression and differences in body mass between *P. reticulata* and their *P. picta* competitors in our experimental competition tanks. *P. reticulata* tended to be smaller than *P. picta* overall, but there was no relationship between aggression difference and body mass differences. Red points are from freshwater tanks and blue points are from brackish water tanks.

Chapter 3

Table.S3.1. ANOVA table for the effect of salinity, competition, and their interaction on the change in body condition in *P. reticulata* over the course of 1 week. Salinity, competition, and their interaction were treated as a fixed factors and round (day the experiment started for each fish) was treated as a covariate (with 3 levels).

Factor	Sum Sq	DF	F-value	P-value
Salinity	0.07487	1	2.9826	0.09518
Competition	0.03372	1	1.3433	0.25624
Salinity: Competition	0.10768	1	4.2894	0.04768
Round	0.07312	2	1.4564	0.25020

Table. S3.2. Least-squared means contrasts for the model from Table S3 in which comparisons between each salinity are made for each level of competition. Statistics reflect a Tukey adjustment for multiple comparisons.

Contrast	Competition	Contrast Estimate	SE	df	T-ratio	P-value
F-B	Interspecific	0.2240	0.0869	28	2.577	0.0155
F-B	Intraspecific	-0.0209	0.0797	28	-0.262	0.7949

Table.S3.3. Repeated measures ANOVA table for the effect of salinity, competition, and their interaction on the change in body condition in *P. reticulata* over the course of 3 week. Salinity, competition, and their interaction were treated as a fixed factors, round (day the experiment started for each fish) was treated as a covariate (with 3 levels), and fish ID as a random factor.

Factor	Sum Sq	Mean Sq	NumDf	DenDF	F-value	P-value
Salinity	0.061270	0.061270	1	20.090	6.1671	0.02196
Competition	0.004513	0.004513	1	20.187	0.4542	0.50797
Salinity: Competition	0.072608	0.072608	1	19.982	7.3083	0.01368
Round	0.008827	0.008827	1		0.8885	0.35714

Table. S3.4. Least-squared means contrasts for the model from Table S3 in which comparisons between each salinity are made for each level of competition. Statistics reflect a Tukey adjustment for multiple comparisons.

Contrast	Competition	Contrast Estimate	SE	df	T-ratio	P-value
F-B	Interspecific	0.3784	0.1170	20.3	3.234	0.0041
F-B	Intraspecific	-0.0179	0.0871	19.5	-0.205	0.8396

Table.S3.5. ANOVA table for the effect of salinity on behavioral dominance over the course of one week. Salinity was treated as a fixed factor and fish ID as a random factor.

Factor	Sum Sq	Mean Sq	NumDf	DenDF	F-value	P-value
Salinity	335.98	335.98	**1**	**13.986**	**5.7123**	**0.03148**

Table.S3.6. Table summarizing the GO results from ShinyGo. The results are broken up by whole module and by integral genes within each module. Corrected FDR values are in parentheses by each significantly enriched GO term.

Module	Biological Process (FDR)	Cellular Component (FDR)	Molecular Function (FDR)	KEGG Pathway (FDR)
Turquoise	Cellular metabolic process (0.0012) Response to abiotic stimulus (0.01) Regulation of triglyceride catabolic process (0.01) Positive regulation of triglyceride catabolic process (0.01) Regulation of lipid metabolic process (0.01) Regulation of metabolic process (0.01) Triglyceride catabolic process (0.01) Protein modification process (0.01) Cellular macromolecule metabolic process (0.01) Positive regulation of lipid metabolic process (0.01) Alcohol biosynthetic process (0.01) Neutral lipid catabolic process (0.01) Acylglycerol catabolic process (0.01) Positive regulation of lipid catabolic process (0.01) Regulation of primary metabolic process (0.01) Regulation of triglyceride metabolic process (0.01) Positive regulation of triglyceride metabolic process (0.01) Cellular protein modification process (0.01) Cellular protein metabolic process (0.0143) Response to oxygen-containing compound (0.0143) Response to temperature stimulus (0.0144) Regulation of cellular metabolic process (0.0163) Glycerolipid catabolic process (0.0163) Insulin-like growth factor receptor signaling pathway (0.0163) Regulation of lipid catabolic process (0.0163) Biological regulation (0.0163) Regulation of response to reactive oxygen species (0.0163) Protein phosphorylation (0.0175) Macromolecule modification (0.0182) Regulation of biological process (0.0226)	NA	Neurotransmitter transporter activity (0.0003) Neurotransmitter:sodium symporter activity (0.0003) Solute:sodium symporter activity (0.0007) Enzyme inhibitor activity (0.001) Solute:cation symporter activity (0.0019) Protein serine/threonine kinase activity (0.0039) Cysteine-type endopeptidase inhibitor activity (0.0043) Organic cyclic compound binding (0.0043) Heterocyclic compound binding (0.0043) Ion binding (0.0061) Symporter activity (0.0069) Kinase activity (0.0085) Insulin-like growth factor I binding (0.0085) Insulin-like growth factor II binding (0.0085) Cysteine-type endopeptidase inhibitor activity involved in apoptotic process (0.0085) Cysteine-type endopeptidase regulator activity involved in apoptotic process (0.0085) Sodium ion transmembrane transporter activity (0.0105) Endopeptidase inhibitor activity (0.0107) Phosphotransferase activity, alcohol group as acceptor (0.0107) Histone-lysine N-methyltransferase activity (0.0107) Adenyl nucleotide binding (0.0107) Adenyl ribonucleotide binding (0.0107) Histone methyltransferase activity (0.0107) Endopeptidase regulator activity (0.0107) ATP binding (0.0118) Lysine N-methyltransferase activity (0.0121) Protein-lysine N-methyltransferase activity (0.0121) Transferase activity, transferring phosphorus-containing groups (0.0129) Protein kinase activity (0.0142) Peptidase inhibitor activity (0.0144)	Autophagy (0.0036) FoxO signaling pathway (0.0159) Mitophagy (0.0313) MTOR signaling pathway (0.0313)
Brown	Alanyl-tRNA aminoacylation (0.0191) Cellular divalent inorganic cation homeostasis (0.0396) Cellular calcium ion homeostasis (0.0396) Regulation of cytosolic calcium ion concentration (0.0465) Calcium ion homeostasis (0.0465) Divalent inorganic cation homeostasis (0.0465)	NA	Protein-L-isoaspartate (D-aspartate) O-methyltransferase activity (0.0113) Alanine-tRNA ligase activity (0.0113) Carboxyl-O-methyltransferase activity (0.026) Protein carboxyl O-methyltransferase activity (0.026)	NA
Green	Actin filament polymerization (0.0447) Regulation of actin filament length (0.0447) Regulation of actin filament polymerization (0.0447) Regulation of protein polymerization (0.0447) Regulation of protein complex assembly (0.0447) Regulation of actin filament organization (0.0447) Regulation of actin polymerization or depolymerization (0.0447) Regulation of actin cytoskeleton organization (0.0454) Regulation of actin filament-based process (0.0454) Protein polymerization (0.0454) Regulation of supramolecular fiber organization (0.0454) Actin polymerization or depolymerization (0.0454)	Mitochondrial membrane (0.0001) Organelle envelope (0.0001) Envelope (0.0001) Mitochondrial membrane part (0.0001) Mitochondrial envelope (0.0001) Mitochondrial inner membrane (0.0001) Organelle inner membrane (0.0001) Mitochondrial part (0.0002) Inner mitochondrial membrane protein complex (0.0002) Mitochondrial protein complex (0.0006) Mitochondrion (0.0008) Organelle membrane (0.0011)	NA	Oxidative phosphorylation (0.0153) Valine, leucine and isoleucine degradation (0.0153) Butanoate metabolism (0.0153) Metabolic pathways (0.0153) PPAR signaling pathway (0.0153) Pentose phosphate pathway (0.031) Fatty acid elongation (0.031) Beta-Alanine metabolism (0.031) Fatty acid degradation (0.0352) Tryptophan metabolism (0.0393) Fanconi anemia pathway (0.0433) Oxidative phosphorylation (0.0153)
Red	Lymphoid lineage cell migration (0.0074) Lymphoid lineage cell migration into thymus (0.0074)	NA	NA	Adipocytokine signaling pathway (0.0261)
Turquoise (integral genes)	Cellular metabolic process (0) Cellular macromolecule metabolic process (0) Response to abiotic stimulus (0.0004) Nitrogen compound metabolic process (0.0004) Macromolecule metabolic process (0.0007) Regulation of biosynthetic process (0.0008) Regulation of gene expression (0.0008) Regulation of nucleobase-containing compound metabolic process (0.0008) Regulation of metabolic process (0.0008) RNA biosynthetic process (0.0008) Protein modification process (0.0008) Regulation of RNA metabolic process (0.0008) Nucleic acid-templated transcription (0.0008)	Nucleus (0.0152)	DNA-binding transcription factor activity (0.0061) Transcription regulator activity (0.0061) Insulin receptor binding (0.0216) Neurotransmitter transporter activity (0.0302) Neurotransmitter:sodium symporter activity (0.0302) Heat shock protein binding (0.0304) Unfolded protein binding (0.0304) Solute:sodium symporter activity (0.0339) Zinc ion binding (0.0366) Enzyme inhibitor activity (0.0458) Solute:cation symporter activity (0.0458)	FoxO signaling pathway (0.0117) Autophagy (0.027) Protein processing in endoplasmic reticulum (0.0291) Arginine biosynthesis (0.0462)

	Regulation of nucleic acid-templated transcription (0.0008) Regulation of RNA biosynthetic process (0.0008) Transcription, DNA-templated (0.0008) Regulation of transcription, DNA-templated (0.0008) Cellular protein modification process (0.0008) Regulation of primary metabolic process (0.0008) Regulation of macromolecule biosynthetic process (0.0011) RNA metabolic process (0.0011) Macromolecule modification (0.0011) Regulation of macromolecule metabolic process (0.0011) Regulation of cellular macromolecule biosynthetic process (0.0011) Regulation of cellular biosynthetic process (0.0012) Nucleobase-containing compound biosynthetic process (0.0012) Organic substance biosynthetic process (0.0013) Regulation of nitrogen compound metabolic process (0.0014) Aromatic compound biosynthetic process (0.0015) Nucleic acid metabolic process (0.0015)			
Brown (integral genes)	NA	NA	NA	Protein processing in endoplasmic reticulum (0.02) Herpes simplex virus 1 infection (0.02) Endocytosis (0.0423) RNA transport (0.0459) Phagosome (0.0459)
Green (integral genes)	Cell proliferation (0.0334)	Mitochondrial envelope (0.0046) Mitochondrial membrane (0.0046) Organelle envelope (0.0046) Envelope (0.0046) Mitochondrial part (0.0046) Inner mitochondrial membrane protein complex (0.0057) Mitochondrion (0.0063) Organelle membrane (0.0065) Mitochondrial membrane part (0.0065) Mitochondrial protein complex (0.0072) Mitochondrial inner membrane (0.0093) Organelle inner membrane (0.0094) Membrane protein complex (0.0287)	NA	NA
Red (integral genes)	NA	NA	NA	NA

Table.S3.7. Table summarizing the GenClip 3.0 pathway analysis results. “Hit” refers to the number of genes leading to the significant enrichment of that pathway, “total” refers to the total number of genes in that pathway, “gene list” refers to the “hit” genes, and “source” refers to the database that supports the enriched pathway.

Pathway	Hit/Enrichment Score	Total	P-Value	Q-Value	Gene List	Source
Cluster1	Enrichment Score: 2.76					
MECP2_and Associated Rett Syndrome (Wikipathways)	3	63	0.000396	0.011921	FKBP5;SGK1;YBX1	Wikipathways
Transcriptional_Regulation by MECP2 (Wikipathways)	2	30	0.002132	0.022389	FKBP5;SGK1	Wikipathways
Glucocorticoid_receptor regulatory network (PID)	3	80	0.000798	0.011921	FKBP5;KMT5B;SGK1	PID
IL2 (NetPath)	2	76	0.013086	0.042278	SGK1;YBX1	NetPath
Cluster2	Enrichment Score: 2.35					
Signaling_mediated by p38-alpha and p38-beta (PID)	2	35	0.002896	0.024326	DDIT3;HBP1	PID
Validated_transcriptional targets of TAp63 isoforms (PID)	2	55	0.007025	0.029504	HBP1;OGG1	PID
Cluster3	Enrichment Score: 2.21					
HSP90_chaperone cycle for steroid hormone receptors (SHR) (Reactome)	2	19	0.000851	0.011921	DNAJA1;FKBP5	Reactome
Cellular_responses to stress (Reactome)	3	345	0.043849	0.098238	DNAJA1;FKBP5;ST13	Reactome
Single1	Enrichment Score: 2.09					
Lysine_degradation - Homo sapiens (human) (KEGG)	2	59	0.008048	0.030729	HADHA;KMT5B	KEGG
Cluster4	Enrichment Score: 2.04					
Metabolism_of polyamines (Reactome)	2	40	0.003769	0.026386	AMD1;ARG2	Reactome
Arginine_and proline metabolism - Homo sapiens (human) (KEGG)	2	50	0.005836	0.029504	AMD1;ARG2	KEGG
Arginine_Proline metabolism (INOH)	2	55	0.007025	0.029504	AMD1;ARG2	INOH
Metabolism_of amino acids and derivatives (Reactome)	3	342	0.042908	0.098238	AMD1;ARG2;ASPA	Reactome
Cluster5	Enrichment Score: 1.86					
FoxO_family signaling (PID)	2	50	0.005836	0.029504	SGK1;ZFAND5	PID
mTOR_signaling pathway (PID)	2	65	0.009701	0.033952	DDIT4;SGK1	PID
mTOR_signaling pathway - Homo sapiens (human) (KEGG)	2	151	0.04678	0.098238	DDIT4;SGK1	KEGG
Single2	Enrichment Score: 1.44					
DNA_Repair (Reactome)	3	320	0.036326	0.098238	OGG1;RAD52;RNF111	Reactome
Single3	Enrichment Score: 1.44					
TGF-beta_Signaling Pathway (Wikipathways)	2	132	0.03668	0.098238	LIMK2;RNF111	Wikipathways
Cluster6	Enrichment Score: 1.35					
PI3K-Akt_Signaling Pathway (Wikipathways)	3	340	0.042286	0.098238	DDIT4;GNG7;SGK1	Wikipathways
PI3K-Akt_signaling pathway - Homo sapiens (human) (KEGG)	3	354	0.046736	0.098238	DDIT4;GNG7;SGK1	KEGG

For Tables S3.8-S3.12, visit https://datadryad.org/stash/share/fF0tGhE3JtwXnkmlU0OEZ4h9B9e3AGuU6P3-AfcwduA. for access to the large data sets described.

Table.S3.8. A list of Salinity Transition Genes identified from the DeSeq2 differential expression analysis. Output from the analysis is included as well as the whether the gene was

included because it was differentially expressed between the interspecific salinity treatments (INTER), intraspecific salinity treatments (INTRA), or both (Overlap) (see “Comp” column).

Table.S3.9. A list the genes included in the brown module and whether the gene was identified as an integral gene within this module.

Table.S3.10. A list the genes included in the green module and whether the gene was identified as an integral gene within this module.

Table.S3.11. A list the genes included in the red module and whether the gene was identified as an integral gene within this module.

Table.S3.12. A list the genes included in the turquoise module and whether the gene was identified as an integral gene within this module.

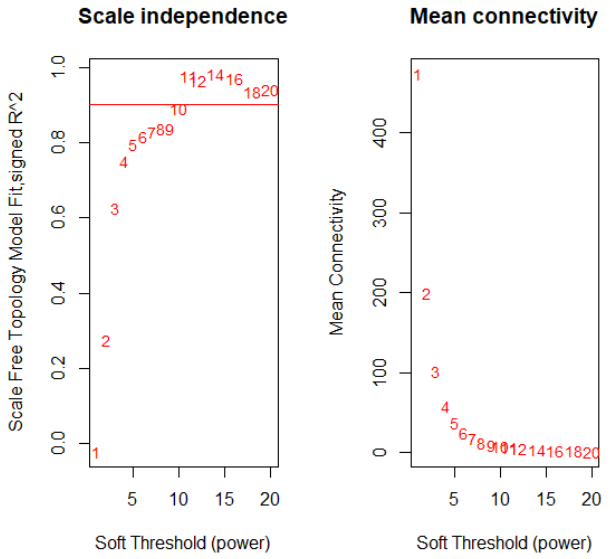

Figure. S3.1. Graphs of the diagnostic tests used to choose the soft-thresholding value of k=10. A value of 10 was chosen because it was the first value that had a scale free topology fit close to a R^2 value of 0.9 and a low mean connectivity. For more details on this process, please consult the documentation for WGCNA (https://rdrr.io/cran/WGCNA/) and the provided tutorials for WGCNA.

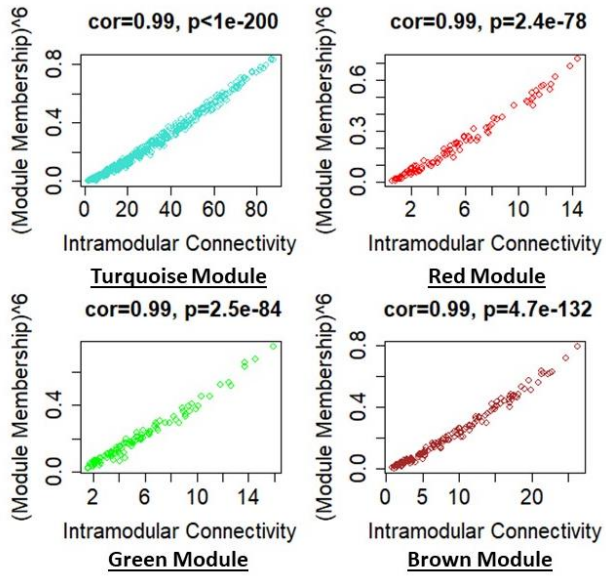

Figure. S3.2. A figure displaying the correlation between module membership and intramodular connectivity for each of the modules that significantly correlated with aggressive behaviors. The near perfect correlation between these two measures meant that using module membership as one of the selection criteria for identifying integral genes within each module also meant selecting genes with high numbers of intramodular connections (a more classic integral or hub gene selection criteria).

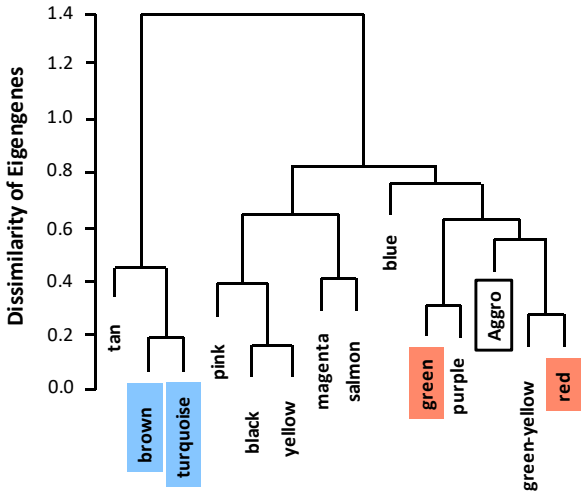

Figure S3.3. A dendrogram displaying the relatedness of the modules' eigengenes to the other modules and aggression ("Aggro"). Modules closer to one another have more correlated eigengenes than modules further from one another (dissimilarity= 1 minus the correlation of the eigengenes). Modules significantly correlated with aggression are highlighted (negatively correlated modules in blue, positively correlated modules in red).

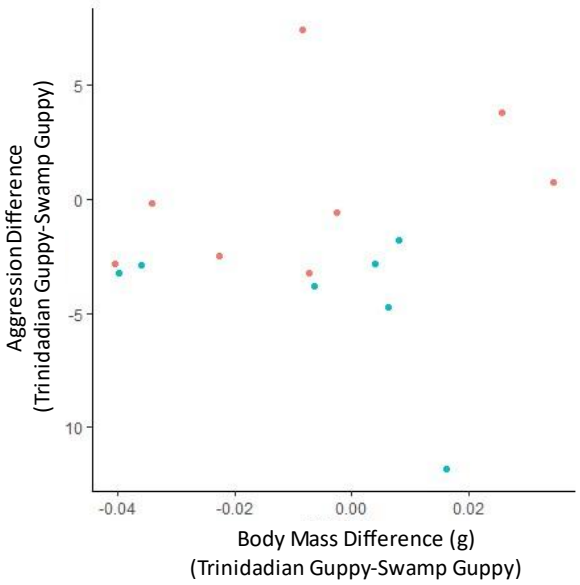

Figure S3.4. A plot showing the relationship between differences in aggressive behavior counts and differences in body mass between *P. reticulata* and their *P. picta* competitors in our experimental competition tanks. *P. reticulata* tended to be smaller than *P. picta* overall, but there was no relationship between aggression difference and body mass differences. Red points are from freshwater tanks and blue points are from brackish water tanks.

Chapter 4

Table S4.1. A matrix of pairwise F_{ST} values between all populations. Naming of populations is coded as "River_Salinity" with CAP=Caparo, CAR=Caroni, CUN=Cunupia, F=freshwater, L=low salinity, M=medium salinity, H= high salinity. Some values are negative as Weir and Cockerham's F_{ST} corrects for sample size, but these values should be interpreted as 0.0.

	CAP_F	CAP_M	CAP_L	CAP_H	CAR_F	CAR_M	CAR_L	CAR_H	CUN_F	CUN_L	CUN_H
CAP_F	NA	0.026618	0.049653	0.021551	-0.0252	0.015283	0.036259	-0.00102	-0.00405	0.041931	-0.00275
CAP_M	0.026618	NA	0.000597	-0.00017	0.00224	0.108767	0.000396	-0.00114	-0.00233	0.00027	0.076519
CAP_L	0.049653	0.000597	NA	0.002029	0.013603	0.129667	0.000687	0.004431	0.002487	0.000193	0.087163
CAP_H	0.021551	-0.00017	0.002029	NA	-0.00067	0.105722	0.000581	-0.00258	-0.00355	0.001258	0.075072
CAR_F	-0.0252	0.00224	0.013603	-0.00067	NA	0.042348	0.007236	-0.01298	-0.01474	0.00992	0.017111
CAR_M	0.015283	0.108767	0.129667	0.105722	0.042348	NA	0.11612	0.077956	0.075674	0.12153	-0.0269
CAR_L	0.036259	0.000396	0.000687	0.000581	0.007236	0.11612	NA	0.001279	6.18E-06	0.000381	0.080072
CAR_H	-0.00102	-0.00114	0.004431	-0.00258	-0.01298	0.077956	0.001279	NA	-0.01199	0.002619	0.046358
CUN_F	-0.00405	-0.00233	0.002487	-0.00355	-0.01474	0.075674	6.18E-06	-0.01199	NA	0.000846	0.041324
CUN_L	0.041931	0.00027	0.000193	0.001258	0.00992	0.12153	0.000381	0.002619	0.000846	NA	0.082491
CUN_H	-0.00275	0.076519	0.087163	0.075072	0.017111	-0.0269	0.080072	0.046358	0.041324	0.082491	NA

Table S4.2. CV values from our ADMIXTURE results for k values 1-6. A k value of 2 was favored (lowest CV value).

K	**CV**

1	0.54072
2	0.50783
3	0.51924
4	0.53209
5	0.5479
6	0.56217

Tables S4.3-4.33. Visit https://doi.org/10.5061/dryad.k0p2ngf7k to access the large tables containing the full lists of the genes found from all the outlier tests and all the results from GO analyses. The online data sheet includes a meta data sheet explaining how the data is filed.

Table S4.34. Full results from our partial RDA analysis from SNPs from only neutral loci. No model was significant and due to some collinearity between explanatory factors confounded inertia was negative.

Name	Model	Constrained Inertia	Adjusted R2	P-value - from ANOVA	Proportion of explainable variance	Proportion of total variance (constrained/total)
Full model	F ~ RD + salinity + IO	261.00	0.27	0.29	1.00	0.34
Pure river distance (RD)	F ~ RD + | (salinity + IO)	92.00	0.29	0.28	0.35	0.12
Pure salinity	F ~ salinity | (RD + IO)	84.00	0.27	0.25	0.32	0.11
Pure iso-osmotic (IO)	F ~ IO | (RD + salinity)	112.00	0.21	0.23	0.43	0.15
Confounded	RD/salinity/IO	-27.00			-0.10	
Total unexplained	from full model	502.00				
Total inertia	from full model	763.00				

Table S4.35. Full results from our partial RDA analysis from SNPs from only outlier loci. No model was significant and due to some collinearity between explanatory factors confounded inertia was negative.

Name	Model	Constrained Inertia	Adjusted R2	P-value - from ANOVA	Proportion of explainable variance	Proportion of total variance (constrained/total)
Full model	F ~ RD + salinity + IO	256.00	0.060	0.27	1.00	0.34
Pure river distance (RD)	F ~ RD + | (salinity + IO)	87.00	0.030	0.31	0.34	0.12
Pure salinity	F ~ salinity | (RD + IO)	77.00	0.010	0.27	0.30	0.10
Pure iso-osmotic (IO)	F ~ IO | (RD + salinity)	102.00	0.050	0.22	0.40	0.13
Confounded	RD/salinity/IO	-10.00			-0.04	
Total unexplained	from full model	498.00				
Total inertia	from full model	754.00				

Table S4.36. ANOVA table for the effect of salinity, population, and their interaction on juvenile growth rate in F2 common garden *P. picta*.

Factor	Sum Sq	Mean Sq	NumDf	DenDF	F-value	P-value
Salinity	3.5101	3.5101	1	70.379	10.4742	0.0018
Population	0.0720	0.0720	1	25.698	0.2147	0.0647
Salinity:Population	0.0001	0.0001	1	70.379	0.0002	0.9878

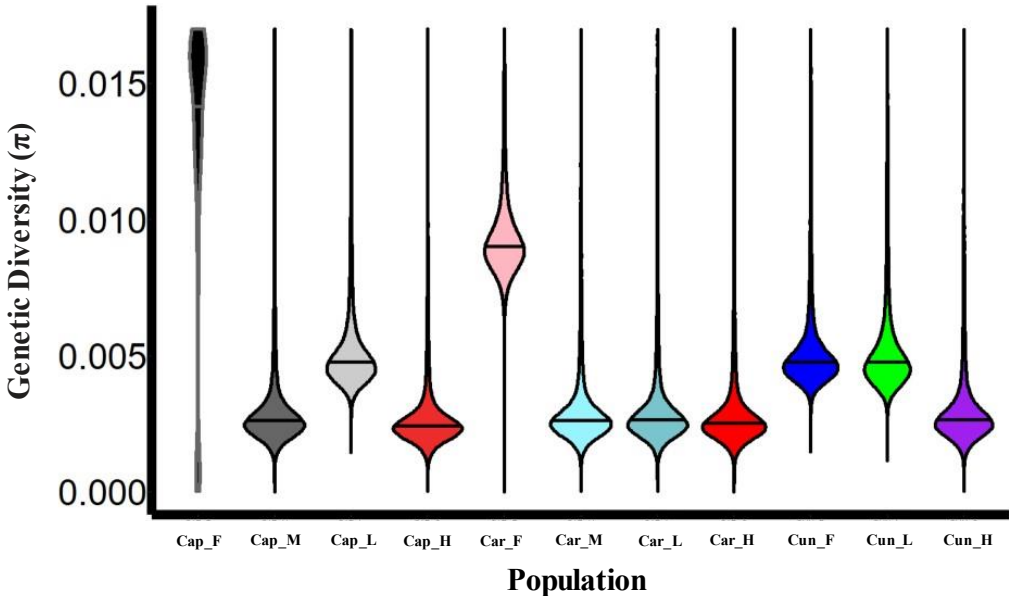

Figure S4.1. A violin plot displaying the genetic diversity (π) of each population. Naming of populations is coded as "River_Salinity" with CAP=Caparo, CAR=Caroni, CUN=Cunupia, F=freshwater, L=low salinity, M=medium salinity, H= high salinity. There is a trend for freshwater and low salinity populations to have slightly elevated values of π.

Table S4.37. Results from the RDA used to identify outlier loci in our GEA test in which salinity was the only explanatory variable. The model was significant (P=0.046) and had an adjusted R^2 value of 0.105.

Type	Inertia	Adjusted R2	P-value - from ANOVA	Proportion of total variance (constrained/total)
Constrained	5.82e+04	0.105	0.046*	0.19
Unconstrained	2.42e+05			0.81
Total	3.00e+05			1.00

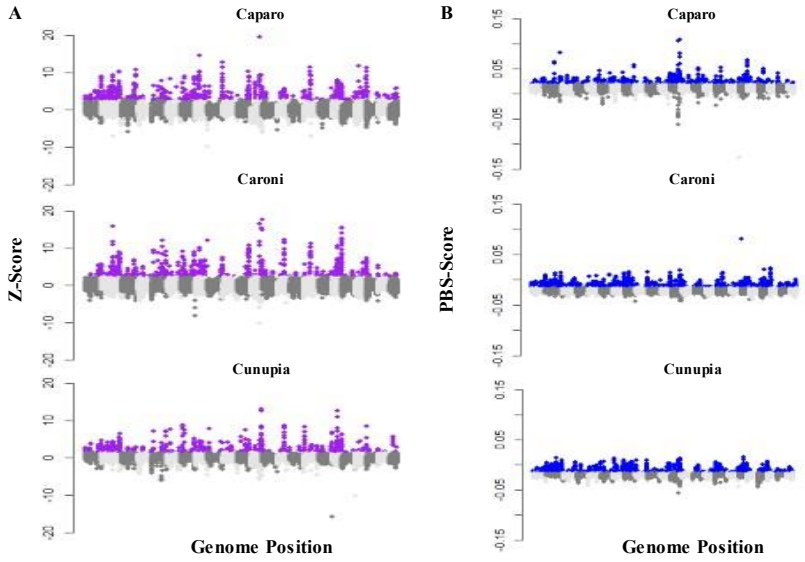

Figure S4.2. Manhattan plots for the z test (A) and PBS outlier (B) tests for each river. 50kb loci are organized by chromosome (alternating black and grey regions) and chromosome position. Highlighted loci (purple in A, blue in B) represent 1% outlier loci for their respective test.

Table S4.38. A table with the latitude and longitude coordinates for each samplings site. Naming of the sites is coded as "River_Salinity" with CAP=Caparo, CAR=Caroni, CUN=Cunupia, F=freshwater, L=low salinity, M=medium salinity, H= high salinity.

Population	Latitude	Longitude
CAP_F	10.51732	-61.43181
CAP_M	10.5249	-61.45079
CAP_L	10.52422	-61.44955
CAP_H	10.52484	-61.46552
CAR_F	10.61917	-61.42662
CAR_M	10.62124	-61.46574
CAR_L	10.6204	-61.45711
CAR_H	10.62535	-61.47439
CUN_F	10.56218	-61.41727
CUN_L	10.56214	-61.43772
CUN_H	10.56213	-61.44286