ADAPTING TO SALINITY: THE EFFECTS OF SALINITY ON POPULATION STRUCTURE AND OFFSPRING SURVIVAL IN *LUCANIA PARVA*

BY

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THESIS

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ABSTRACT

Adaptation to salinity is an important driving force in the evolution in teleost fishes. Some speciose groups such as minnows and characids are found predominantly in fresh water, while other groups such as tunas and wrasses are found predominantly in marine habitats. Euryhaline groups, such as killifish, contain freshwater species, marine species, and species that can occur in fresh, brackish, and marine conditions. These groups are powerful systems for studying adaptation to salinity as they allow for the comparison of close relatives who differ in salinity tolerance. In chapter 1, I review the biology of *Lucania* killifish. *Lucania* contains three species, one of which is a freshwater species (*L. goodei*) and another of which is euryhaline (*L. parva*). A third species (*L. interioris*) is endemic to a small region in Mexico and is not considered in this thesis. Previous studies on *L. goodei* and *L. parva* suggest that salinity has dramatic effects on life-history, ecology, physiology, and genetic differentiation at the between-species level. Upon salinity transfer, the two species differ in gene expression in critical osmoregulatory genes. An examination of F_{ST} outliers suggests that the two species differ in many genes related to osmoregulation, but that they also possess high levels of differentiation between genes involved in reproduction and spermatogenesis. Within *L. parva*, preliminary work indicated that freshwater-saltwater population pairs also possessed elevated levels of differentiation in loci related to osmoregulation.

In chapter 2, I used RAD-Seq data from 10 populations across Florida to examine the levels of population structure between freshwater and saltwater populations and the effects of distance on population-wide F_{ST}. Here, I found good evidence that differences in salinity increase F_{ST} beyond what would be expected from the effects of distance alone.

In chapter 3, I describe a laboratory experiment that tests for both local adaptation and for maternal effects as a function of salinity. Early development is a critical life stage. From their earliest moments in life, embryos cannot regulate their ion and water levels because the physiological traits needed for active osmoregulation have not yet developed. Instead, embryos rely on the properties of the egg and maternal provisioning to maintain proper ion and water levels. Hence, this stage of development is ripe for maternal effects (either genetic or environmental) that influence offspring survival as a function of salinity. In chapter 3, I describe the results of an experiment where I performed within population crosses for a freshwater and a saltwater population from the Wakulla River drainage. In the experiment, I considered the effects of population of origin (fresh versus salt) and the effects of spawning salinity (the salinity in which spawning pairs were housed) and rearing salinity (the salinity in which eggs and fry were kept). Hence, the experiment allows me to examine the effects of population of origin and parental salinity environment on subsequent survival as a function of salinity. Here, I found little evidence for local adaptation as a function of salinity. Maternal effects were present, but the nature of the pattern did not suggest that they were adaptive. I suggest that other life-history stages such as over-winter survival, perhaps in the presence of intraspecific competition, should be assessed.

My thesis indicates that there is evidence for heightened genetic divergence between freshwater and saltwater populations, yet we do not know precisely how these effects emerge. Salinity may affect multiple life-history stages (i.e., growth, survival to adulthood, over-winter survival) beyond the ones examined in this thesis. Salinity may also affect multiple aspects of ecology, including community composition (i.e., potential competitors, predators, and prey items), which may create parallel selection due to ecological demands.

ACKNOWLEDGMENTS

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To each and every one of you: *le hannon*.

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CHAPTER 1: A REVIEW OF SALINITY TOLERANCE IN *LUCANIA*

ABSTRACT

Adaptation to salinity is an important driving force in the evolution in teleost fishes. Some speciose groups such as minnows and characids are found predominantly in fresh water, while other groups such as tunas and wrasses are found predominantly in marine habitats. Euryhaline groups, such as killifish, contain freshwater species, marine species, and species that can occur in fresh, brackish, and marine conditions. These groups are powerful systems for studying adaptation to salinity as they allow for the comparison of close relatives who differ in salinity tolerance. In this chapter, I review the biology of *Lucania* killifish. This groups contains three species, one of which is a freshwater species (*L. goodei*) and another of which is euryhaline (*L. parva*). I review the effects of salinity on life-history, ecology, physiology, and genetic differentiation at the between-species level. I also discuss the current state of knowledge of the effects of salinity at the among population, within species level.

INTRODUCTION

Ion and osmo-regulation (the active regulation of internal ion and water levels at particular levels) is critical in vertebrates to preserve cellular, physiological, and neural processes (Loretz and Bern 1982, Hwang et al. 2011, Kültz 2015). Aquatic organisms face particular challenges because - in the lack of active processes - passive diffusion and osmosis result in internal ion and water levels converging on those of the external environment (Parry 1966, Evans and Claiborne 2008). Most fish species can only tolerate either a freshwater or a saltwater environment but cannot switch between the two due to the opposing osmotic needs and stresses

freshwater and saltwater environments impose (Gunter 1945, 1950, Whitfield 2015, Nelson et al. 2016). Teleost fishes regulate their plasma osmotic concentration to be about one-third that of marine water; thus, fishes in fresh water must compensate for the passive gain of water and loss of ions to the environment by producing high quantities of diluted urine and minimizing renal salt loss, while fishes in salt water must compensate for the passive loss of water and gain of ions to the environment by ingesting salt water and secreting ions out the gills (McCormick 2001, Evans et al. 2005). The physiological mechanisms involved in osmoregulation vary greatly between fresh water and marine fishes (Reviewed in Evans et al. 2005). In stable environments, most fish species are stenohaline, specializing in either fresh water or salt water (Kültz 2015).

Euryhaline species are of note because of their ability to tolerate a broader range in salinity (Whitfield 2015, Kültz 2015, Nelson et al. 2016). Many euryhaline species like salmon and eels are diadromous, meaning they migrate between fresh and saltwater environments (McDowall 1997, Zydlewski and Wilkie 2012). Anadromous species, such as salmon, are spawned in fresh water, then migrate to marine environments before returning to fresh water as adults to breed (McDowall 1997, Björnsson et al. 2011, Nelson et al. 2016). Catadromous species, such as European and North American eels, do the reverse, being spawned in the sea, migrating to fresh water, then returning to the sea to breed (McDowall 1997, Nelson et al. 2016, Cao et al. 2018). In both anadromous and catadromous species, the transition between freshwater and saltwater environments occurs at predictable times during specific life history stages (McDowall 1997, Björnsson et al. 2011, Zydlewski and Wilkie 2012). These fishes typically do not transition rapidly from one salinity habitat to another. Instead, they usually spend prolonged periods of time at the coasts as they undergo elaborate changes to their osmoregulatory system like changes in gill structure, ionocyte composition, hormone levels, and enzyme production

(Björnsson et al. 2011, Zydlewski and Wilkie 2012). For example, anadromous green sturgeon experience an increase in plasma cortisol and a decrease in Na^+/K^+-ATPase activity in the gill lamellae as juveniles prepare to transition into saltwater (Allen et al. 2009). The migrations of diadromous species often mark transitions in life-stages, and osmoregulatory changes are also frequently paired with other morphological, physiological, and behavioral changes associated with other biotic factors (i.e. predators, prey, mates) or abiotic factors (i.e. water flow, oxygen content; Björnsson et al. 2011).

In contrast, other euryhaline species, such as killifish, typically live in environments with less predictable salinity levels that are not linked to specific life-stages (Whitehead 2010, Marshall 2012, Whitfield 2015, Kültz 2015). Generations may be spent in just fresh water or salt water, but there may be times when the salinity changes rapidly. Weather events can cause a sudden and significant changes in salinity (Cardoso et al. 2008). Extreme rain and floods can decrease salinity. Hurricanes can lead to sudden increases in salinity as storm surges push sea water into inland waters (Walker 2001, Illangasekare et al. 2006, Fuller 2008a). Droughts can also increase salinity as evaporation of water increases the concentration of salt (Nielsen and Brock 2009). In order to persist in these conditions, fish have to possess the ability to transition between different behaviors and physiological mechanisms to rapidly accommodate fresh, brackish, or marine habitats (Wood and Marshall 1994, Kültz 2015).

Lucania killifish provide a compelling system to study the evolution salinity tolerance, because close relatives differ in salinity tolerance (Whitehead 2010). There are three species in this genus: *L. parva, L. goodei,* and *L. interioris* (Hubbs and Miller 1965). *Lucania interioris* is an endangered species that is endemic to isolated parts of the Cuatrocienagas region of Mexico (Hubbs 1936). *L. interioris* is often found in highly alkaline (i.e., basic) and slightly to highly

saline water (Miller et al. 2005). I do not consider this species further in this thesis. *L. goodei* is a freshwater species that is very common in Florida with a few populations occurring in Alabama, Georgia, and North and South Carolina (Figure 1.1A; Page and Burr 2011). *L. parva* is extremely euryhaline with populations in fresh, brackish, marine, and hypermarine habitats ranging from Cape Cod to the Atlantic and Gulf coasts (Figure 1.1B; Hubbs and Miller 1965, Miller et al. 2005, Page and Burr 2011). Figure 1.1C shows the joint distribution of these two species in their area of overlap in Florida (Fuller and Noa 2008). *L. goodei* and *L. parva* co-occur in freshwater and mildly brackish sites that occur along the coasts and in the St. John's River in Florida (Fuller and Noa 2008). A review of 1,394 museum records from the University of Florida Museum of Zoology supports these broad habitat designations (Fuller and Noa 2008). For *L. goodei,* 92.7% of populations were classified as freshwater, 6.9% were classified as brackish, and less than 1% as marine. In contrast, for *L. parva,* 23.2% of populations were classified as freshwater, 44.8% were classified as brackish, and 32% were calculated as marine. Hence, the distribution of these two species across Florida is consistent with the classification of *L. goodei* as 'freshwater' and *L. parva* as 'euryhaline'.

These differences in habitat classification are reflected in differences in survival in fresh, brackish, and marine water across various life-history stages. Three different studies have compared survival across different salinities (Fuller et al. 2007, Fuller 2008b, Fuller and Noa 2008). Specifically, those studies measured the proportion of eggs that develop, hatch, and eat food in the larval stages. Figure 1.2A shows the results from Fuller (2008b). The goal here was to evaluate egg hatching and larval survival in salinities ranging from fresh to full strength marine conditions. In this experiment, eggs and larval fish were raised in 0 ppt (fresh water), 10 ppt (the isosmotic point), 20 ppt, and 30 ppt (full strength sea water). Both *L. parva* and *L.*

goodei have high egg hatching success at low and intermediate salinities. However, above the isosmotic point (10 parts per thousand [ppt] salt), hatching success decreases dramatically for *L. goodei* eggs. In contrast, *L. parva* have amazingly high egg hatching success across all salinities. Given high juvenile survival across all salinities, why are *L. parva* found predominantly along the coasts? Why don't they invade interior freshwater sites in Florida? The answer may lay in adult survival. Fuller and colleagues raised both *L. goodei* and *L. parva* in stock tanks that were set at either 0, 2, 4, or 8 ppt salinity. They chose these salinities because they represent the gradient over which the biggest changes in relative abundance occurs, with *L. goodei* in high abundance at 0 ppt and *L. parva* in high abundance in mildly brackish water. The fish had to grow from larvae and overwinter in stock tanks in north Florida. Figure 1.2B shows the results. *L. parva* had its lowest survival at 0 ppt and its high survival at 8 ppt. In contrast, *L. goodei* had high survival at all salinities (0 to 8 ppt). These differences in survival were roughly matched by patterns in adult body size (Figure 1.2C).

In addition to survival, differences in salinity also have dramatic effects on the outcome of competition between *L. goodei* and *L. parva.* Dunson and Travis (1991) set up a simple competition experiment where they measured the growth of individuals maintained in one of two treatments: raised with conspecifics (i.e., their own species) or raised with heterospecifics (i.e., the other species). They also examined the effects of salinity on competition by conducting the treatments in both fresh water (0 ppt) and brackish water (15 ppt). The results are shown in Figure 1.3. The key comparison is how body weight changed while in competition in fresh and brackish water. In fresh water, *L. goodei* had greater weight gain when competing with *L. parva* than when competing with conspecifics. The reverse is true for brackish water. *L. goodei* had greater weight gain when competing with conspecifics than when competing with *L. parva.* For

L. parva, weight gain was greater in brackish water when in competition with *L. goodei* than when in competition with conspecifics. The implication is that *L. goodei* has a competitive advantage over *L. parva* in freshwater and that *L. parva* has a competitive advantage over *L. goodei* in freshwater.

These differences in salinity-dependent survival and competition are the result of differences in physiology, gene expression, and genetic composition between the two species. Preliminary work comparing mechanisms of salinity tolerance between the two species show clear differences in gene expression as a function of salinity (Berdan and Fuller 2012a, Kozak et al. 2014). Ion- and osmo-regulation occur primarily in the gills with secondary help from the guts, kidneys, and skin (McCormick 2001, Evans et al. 2005, Hwang et al. 2011). In fresh water, diffusion favors the loss of ions to the external environment and gain of water (Hwang et al. 2011). In salt water, the opposite happens with ions diffusing into the fish and water leaving via osmosis at the gills (Hwang et al. 2011). Fishes use ion pumps/channels, aquaporin water channels, and cell-cell junctions that create gradients that favor the flow of ions and water in specific tissues against the larger environmental gradient (McCormick 2001, Hwang et al. 2011). For example, activity of Na^+/K^+-ATPase pumps, in particular, seem to increase when transitioning to saltwater, while H^+-ATPase activity is generally increased in freshwater, though there is variation across species (Berdan and Fuller 2012b, Zydlewski and Wilkie 2012). Furthermore, there are multiple forms of these osmoregulatory proteins – such as Na^+/K^+-ATPase isoforms α1a, which is upregulated in freshwater fish and assists with sodium uptake, and isoform α1b, which is upregulated in saltwater and assists with excreting excess sodium (Bystriansky et al. 2007, Helfman et al. 2009). Aquaporin water channels and cell-cell junctions

are responsible for regulating water and solute flow across plasma membranes or between cells, respectively (Edwards and Marshall 2012).

In a two-part investigation, Kozak et al. (2014) performed genomic scans and genome expression profiling to compare osmoregulatory genes and pathways associated with different osmotic environments. First, microarrays designed for the closely related *Fundulus heteroclitus* were used to compare gene expression in *L. goodei* and *L. parva* during a transfer from 0 to 15 ppt salinity. Because the microarray probes were unable to measure expression of different alleles of the same gene, it was assumed that genetic divergence between the microarray probes and transcripts from the *Lucania* species would be uniform. Both *L. goodei* and *L. parva* were maintained in fresh water for two months and then transferred to 15 ppt. They were sampled pre-transfer and then at 6, 24, 72, and 240 hours post-transfer. To account for nucleotide substitutions that were unable to be picked up by the microarray probes, focus was put on expression differences during acclimation. Gene expression differed as a function of species, pre- versus post-transfer, and an interaction between species and transfer. Gene ontology enrichment analyses were performed using the microarray data. Genes selectively expressed in *L. goodei* were not enriched for any gene ontology functions. In contrast, genes selectively expressed in *L. parva* were enriched for the GO category 'ion transport' and included Na^+/K^+ ATPase subunits, serine/threonine protein which regulates several ion channels and pumps, osmotic stress transcription factor, malcavernin which is involved in hyperosmotic induced p38 MAPK activation, inositol monophosphitase, and aquaporins (Kozak et al. 2014). Hence, gene pathways critical to osmoregulation are differentially expressed between the two species.

The second goal of Kozak et al. (2014) was to compare transcriptomes between species and populations. Kozak et al. (2014) sampled *L. parva* from three drainages that allowed for a

comparison between coastal brackish/marine populations and inland freshwater populations (Figure 1.4A). Two of these occurred in Florida (Florida - Atlantic Coast; Florida - Gulf Coast, and Texas). They also sampled *L. goodei* in two Florida populations (North Florida Wakulla River and South Florida Everglades). They sequenced RNA that were pooled from gills and gonads from all populations. This allowed for transcriptomes to be assembled *de novo*, and each population was aligned separately to that reference. From these data, they identified SNPs and calculated F_{ST} values between the two species as well as between populations within species. F_{ST} estimation was also done with Infinium bead genotype data, and genomic SNPs were paired with the correct SNP windows from the pooled analysis. The aligned Infinium genotype data, was then used for GO analyses of outlier loci. Comparisons of transcriptomes between the two species also indicate sequence differences in genes critical to osmoregulation (Kozak et al. 2014). Interestingly, the genes that were differentially expressed in the salinity transfer experiment were not the same as those identified as potential outlier loci from the transcriptome data. Figure 1.4B shows the distribution of F_{ST} values between *L. parva* and *L. goodei.* Between the two species, the average F_{ST} across all SNPs was 0.38, and the distribution of F_{ST} values had a clear 'U' shape with many SNPs having low F_{ST} values, few having an intermediate number, and a substantial portion of SNPs with high F_{ST} values. A GO analysis indicates that reproductive genes (e.g. spermatogenesis genes, estrogen receptors, etc.) were enhanced as were genes for water transport and glucocorticoid signaling, which are important to osmoregulation.

Intriguing patterns emerge at the within-species level when comparing transcriptomes. Figure 1.5 shows the F_{ST} distributions for two of the most distant saltwater populations (Florida Atlantic Coast vs. Texas Gulf Coast) in comparison to freshwater-saltwater pairs in close proximity. The average F_{ST} values between two distant saltwater populations is 0.11 in

comparison to three pairs of freshwater-saltwater populations that are closer geographically to one another and range in F_{ST} values from 0.12 to 0.16. Furthermore, only 0.3% of SNPs had F_{ST} values greater than 0.8 for the geographically distant population pair. In contrast, the freshwater-saltwater population pairs (FL Atlantic, FL Gulf, and TX Gulf) had 4.7%, 1.7%, and 4.9% of SNPs with F_{ST} values greater than 0.8, respectively. There are more highly differentiated SNPs for freshwater-saltwater pairs than for distantly located populations that share the same salinity. An analysis of outlier SNP windows indicates that ion transport genes were enriched and that this included ligand-gated channel activity, ion transmembrane transporter activity, and monovalent inorganic cation transmembrane transporter activity. In addition, genes relating to cell junction and several tight junction proteins were enriched, including claudins (specifically the genes that produce claudin 4, claudin 10, claudin 17), which have been implicated in salinity tolerance in close relatives (Kozak et al. 2014).

In summary, salinity has dramatic effects on the biology of *Lucania. L. goodei* and *L. parva* differ in salinity in tolerance with *L. goodei* located in freshwater habitats and *L. parva* found in freshwater, brackish, and marine habitats. These differences in habitat are reflected in salinity-dependent survival and growth. The effects of salinity transfer on gene expression and physiology differ between the two species. There is good evidence that differential selection as an effect of salinity has resulted in genetic differentiation in the genomes of these two species. Finally, there is some evidence to suggest that differential selection as an effect of salinity has resulted in differentiation between freshwater and saltwater populations at the within species level. The fact that freshwater-saltwater populations have elevated F_{ST} values for genes related to osmoregulation suggests the presence of local adaptation as a function of salinity.

In this thesis, I investigate the extent to which there is evidence for local adaptation between freshwater and saltwater populations of *L. parva.* In chapter 2, I follow up on the initial results of Kozak et al. (2014) with a RAD-Seq study of population structure. I used RAD-Seq data from 10 populations across Florida to examine the levels of population structure between freshwater and saltwater populations and the effects of distance on population-wide F_{ST}. Here, I find good evidence that differences in salinity increase F_{ST} beyond what would be expected from the effects of distance alone.

In chapter 3, I describe a laboratory experiment that tests for both local adaptation and for maternal effects as a function of salinity. Early development is a critical life stage. From their earliest moments in life, embryos cannot regulate their ion and water levels because the physiological traits needed have not yet developed. Instead, embryos rely on the properties of the egg and maternal provisioning to maintain proper ion and water levels. Hence, this stage of development is ripe for maternal effects (either genetic or environmental) that influence offspring survival as a function of salinity. In chapter 3, I describe the results of an experiment where I performed within population crosses for a freshwater and a saltwater population from the Wakulla River drainage. In the experiment, I considered the effects of population of origin (fresh versus salt) and the effects of spawning salinity (the salinity in which spawning pairs were housed) and rearing salinity (the salinity in which eggs and fry were kept). Hence, the experiment allows me to examine the effects population of origin and parental salinity environment on subsequent survival as a function of salinity. Here, I found little evidence for local adaptation as a function of salinity. Maternal effects were present, but the nature of the pattern did not suggest that they were adaptive. I suggest that other life-history stages such as over-winter survival, perhaps in the presence of intraspecific competition, should be assessed.

FIGURES

Figure 1.1. The distribution of (A) bluefin killifish, *Lucania goodei,* (B) rainwater killifish, *Lucania parva*, and (C) the joint distribution of *L. goodei* and *L. parva* in Florida. (A) from Page and Burr (2011), (B) from Miller et al. 2005, (C) from Fuller and Noa (2008).

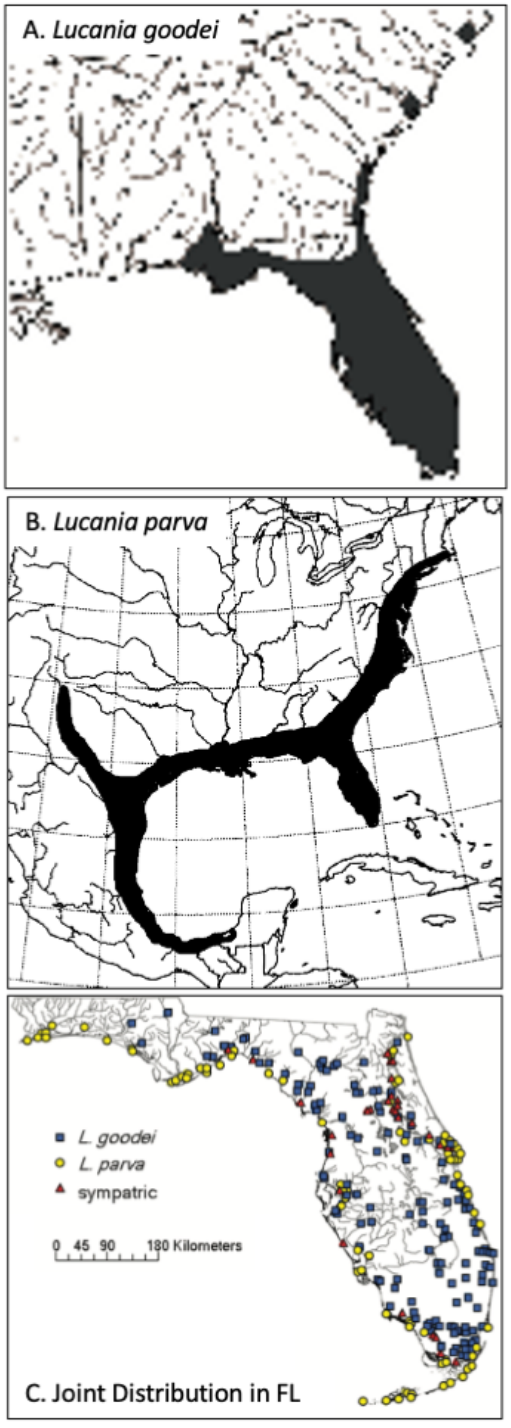


Figure 1.2. Salinity-dependent life-history traits in *L. goodei* and *L. parva.* (A) Hatching success; (B) over-winter survival to adulthood; (C) body size (standard length). Data for (A) are from Fuller (2008b). Data for (B) and (C) are from Fuller et al. (2007).

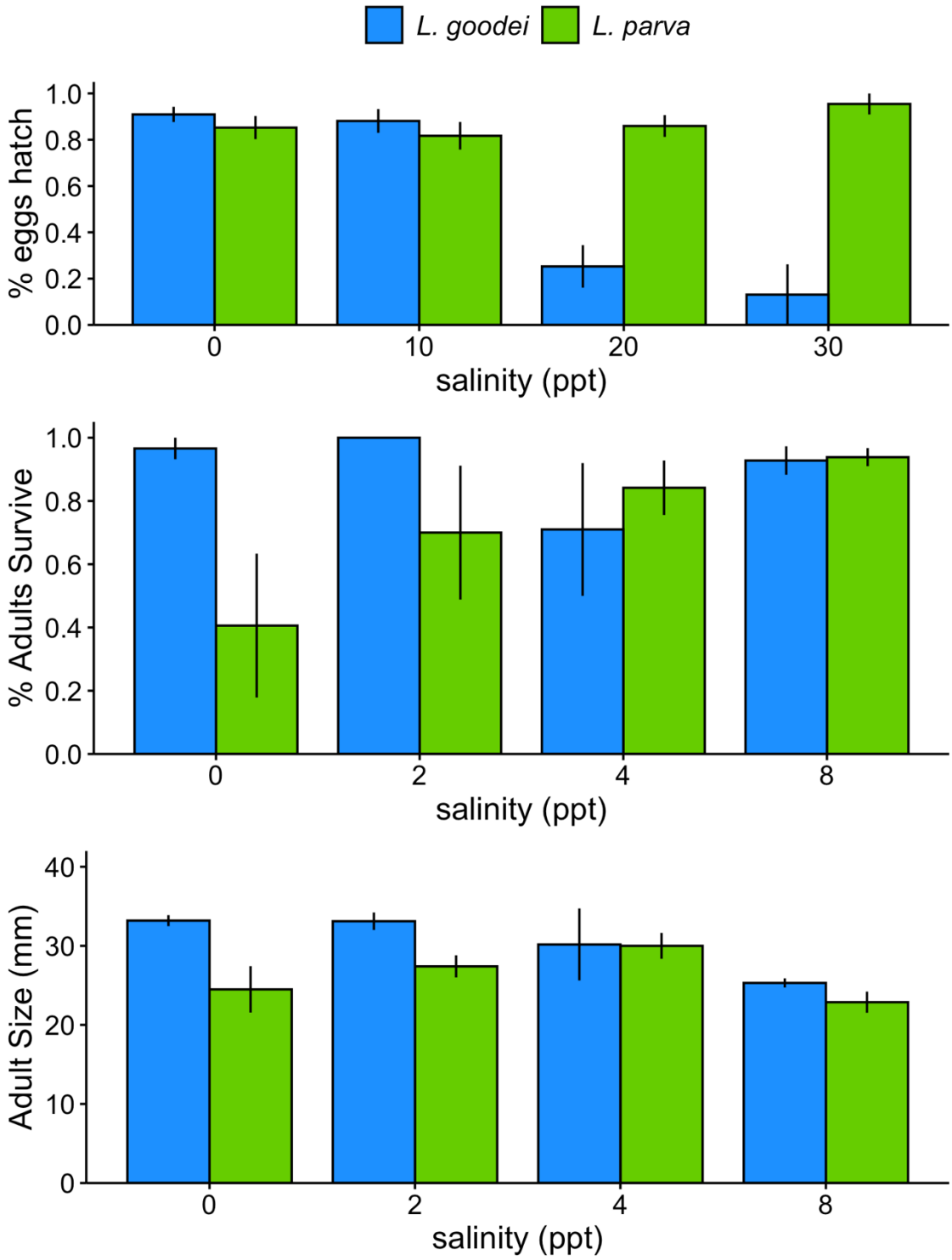


Figure 1.3. Differential effects on salinity on inter-specific competition. Change in body mass as a function of salinity and competition for (A) *L. goodei* and (B) *L. parva.* Data are redrawn from Dunson and Travis (1991).

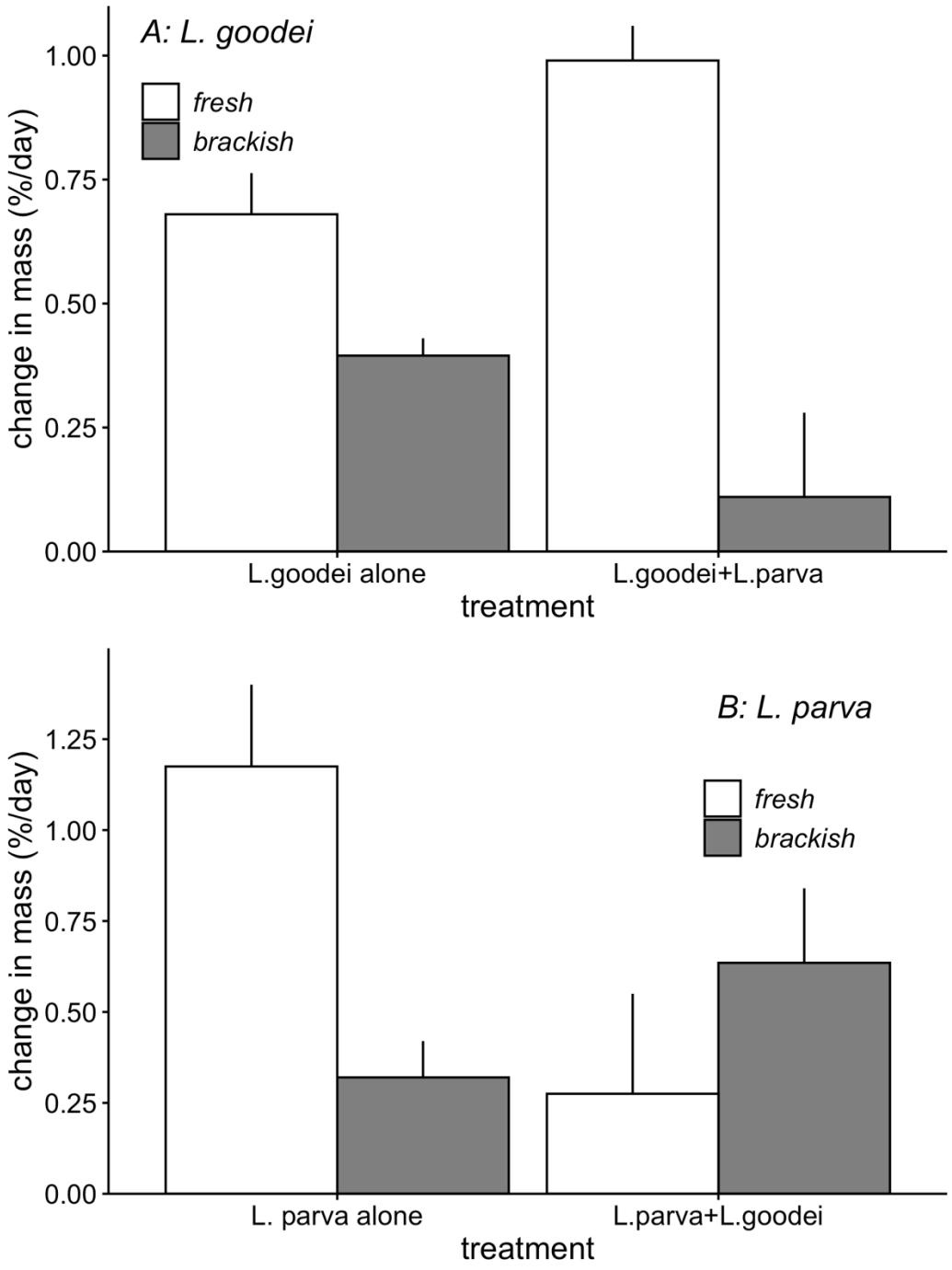
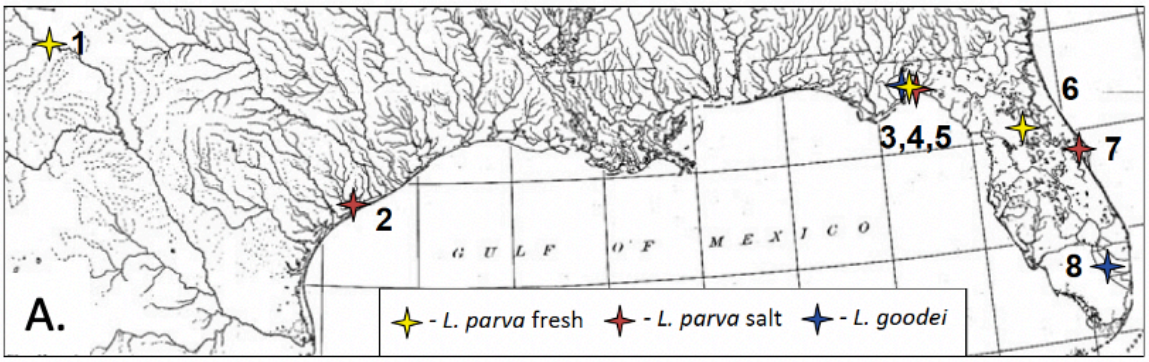


Figure 1.4. (A) Locations of sampling sites of 2 populations of *L. goodei and* 3 freshwater-saltwater population pairs of *L. parva.* (B) F_{ST} distribution between *L. parva* and *L. goodei*. (B) is redrawn from Kozak et al. 2014.



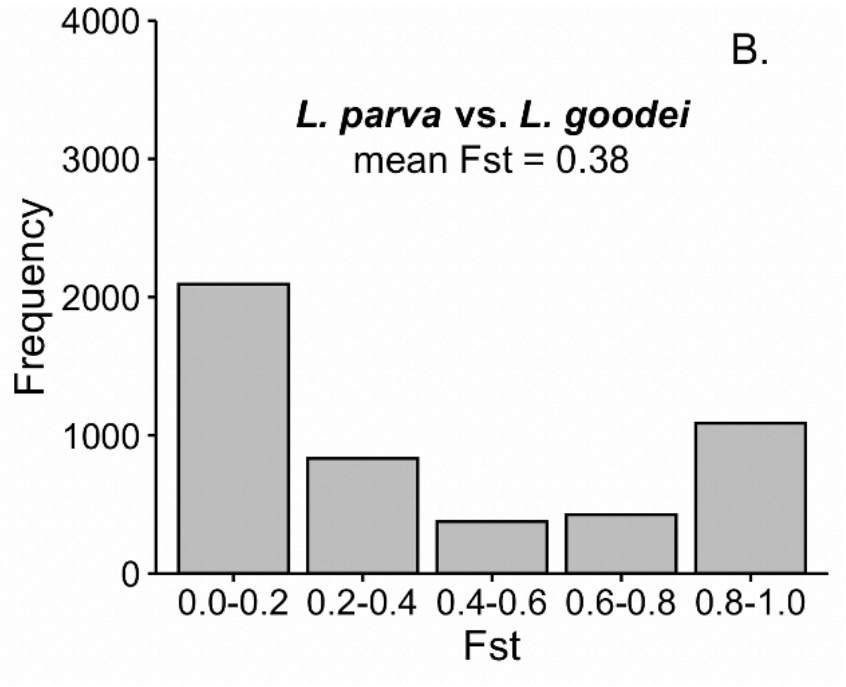
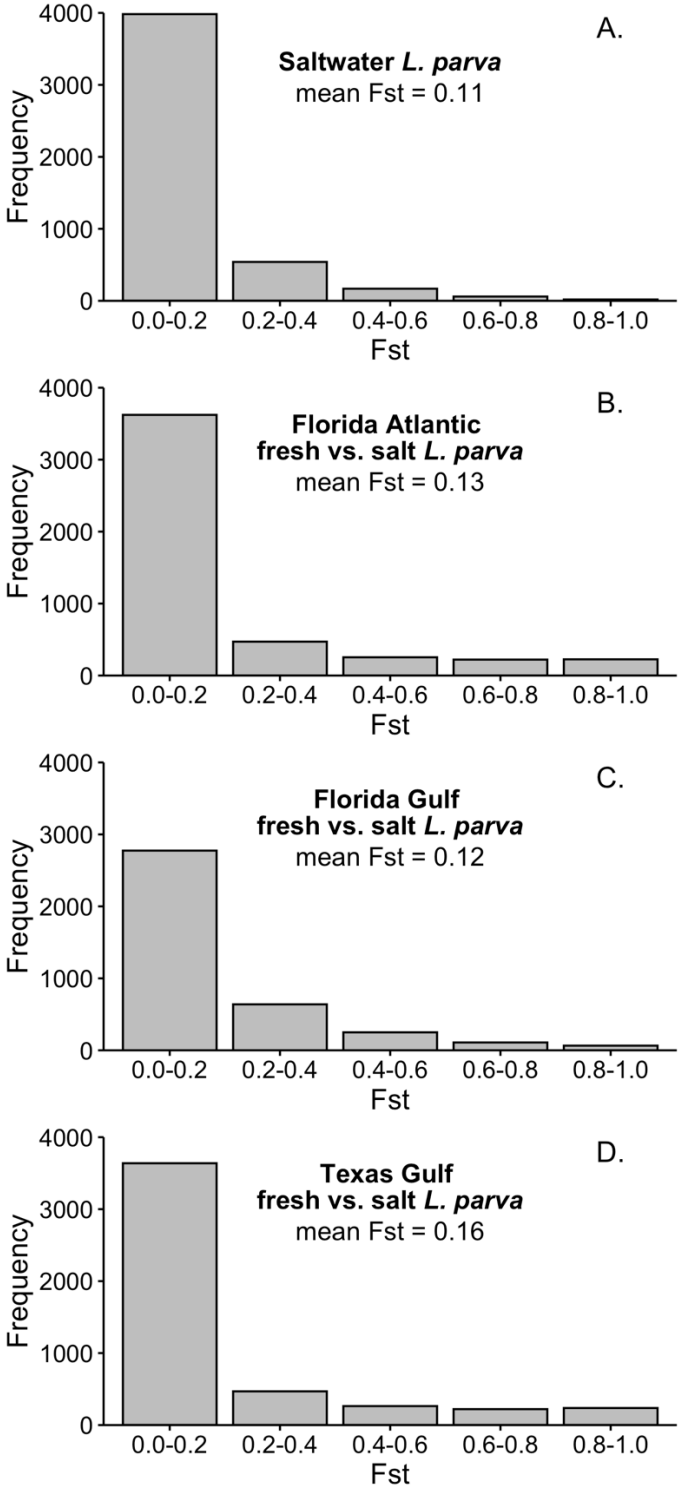


Figure 1.5. F_{ST} values between (A) the two most distant saltwater populations of *L. parva* (Florida Atlantic and Texas Gulf Coast populations) and between freshwater-saltwater population pairs from (B) Florida Atlantic, (C) Florida Gulf Coast, and (D) Texas Gulf Coast. Data are redrawn from Kozak et al. 2014.



CHAPTER 2: AN EXAMINATION OF THE ROLE OF SALINITY ON POPULATION STRUCTURE IN *LUCANIA PARVA*

ABSTRACT

Population structure – the pattern of genetic differentiation among populations within species – is strongly affected by gene flow/dispersal and local adaptation. Among populations, isolation by distance, where more distant populations have higher levels of F_{ST}, is indicative of genetic differentiation due to a lack of gene flow via dispersal. In contrast, isolation by environment, where populations in different habitats have greater genetic differentiation, is a hallmark of isolation via adaptation where immigrants from different environments suffer. In this study, we asked whether differences in salinity lead to genome-wide population structure in the rainwater killifish, *Lucania parva*. Salinity is a critical environmental variable because organisms need to regulate their internal solute and water levels to maintain proper cellular and physiological functions. Freshwater fishes must actively retain salts and expel water, while saltwater fishes must retain water and expel salts. The two demands are diametrically opposed. Most fish groups are predominantly found in either fresh or salt water. Yet, euryhaline fish species can, by definition, occur in a broad range of salinities. Some species in the group Cyprinodontiformes can occur in salinities ranging from fresh water to salt water. *L. parva* can tolerate salinities ranging from 0 to >60 ppt, which is twice the concentration of sea water. We asked whether there was evidence of isolation by distance or isolation by habitat. To do this, we sampled 10 populations (5 fresh, 4 salt, 1 intermediate) of the rainwater killifish, *Lucania parva*, across Florida ranging the east coast of Florida to the Everglades and Florida Keys and along the Gulf Coast to North Florida. We used restriction site-associated DNA (RAD) markers to

determine the effects of drainage, distance between populations, and salinity on F_{ST} values. We found a very strong signature of drainage (east vs. west coast of Florida). We also found a signature of salinity after controlling for the effects of drainage. These data suggest that natural selection as a function of salinity has occurred in *L. parva*.

INTRODUCTION

Population structure – the pattern of genetic differentiation among populations within species – is strongly affected by gene flow/dispersal and local adaptation (Bohonak 1999, Savolainen et al. 2007). Among populations, isolation by distance, where more distant populations have higher levels of F_{ST}, is indicative of genetic differentiation due to a lack of gene flow via dispersal (Britten et al. 1995, Bockelmann et al. 2003). In contrast, isolation by environment, where populations in different habitats have greater genetic differentiation, is a hallmark of isolation via adaptation where immigrants from different environments suffer (Wang and Bradburd 2014). Fishes provide a compelling system for studies of population structure and isolation by distance as their dispersal is limited by the connectivity of water bodies (Planes and Fauvelot 2002, Fullerton et al. 2010). In addition, environmental conditions also can differ dramatically across water bodies in terms of temperature, water flow, water chemistry, and community ecology (Dunson and Travis 1991, Bornette and Puijalon 2011). One particularly important variable is salinity.

Teleost fishes inhabit a wide variety of habitat types and salinity gradients. However, fish assemblage structure changes rapidly from fresh water to marine water, indicating the differing abilities of species to deal with salinity stress imposed by different osmotic environments (Gunter 1945, 1950). In fresh water, teleost fishes must retain ions and keep out excess water,

whereas in marine habitats fishes must retain water but remove excess ions (Evans et al. 2005, Kültz 2015). Entire fish taxa are only found in fresh water or salt water, suggesting the evolutionary transition along a salinity gradient is difficult for many taxa (Lee and Bell 1999, Nelson et al. 2016). Some taxa are able to make this evolutionary transition more readily, containing species in fresh, brackish, and marine environments. While a minority of fish species are not euryhaline, euryhaline species are a notable source of evolutionary diversity (Schultz and McCormick 2012, Nelson et al. 2016). In order to tolerate wide ranges in salinity, euryhaline fish have complex adaptions in a range of ion/osmoregulatory mechanisms involving various organs (e.g. gills, kidneys, and intestines), cell types and machinery (e.g. ion pumps/channels, aquaporin water channels, and cell-cell junctions), enzymes (e.g. ATPase), and life history (McCormick 2001, Evans et al. 2005, Hwang et al. 2011, Berdan et al. 2014, Kozak et al. 2014). For example, when adjusting to freshwater, the hormone prolactin can lead to decreasing the permeability of gill, kidney, and intestinal membranes to water while increasing the uptake of sodium and chloride (Evans et al. 2005, Helfman et al. 2009). Cell-cell junctions become "tighter" to limit the loss of ions and H^+-ATPase activity increases to maintain acid-base balance (Scott et al. 2005, Kültz 2015). Such changes are largely due to osmosensors like TRPV4 (ion) channels or the calcium sensing receptor protein and their signaling pathways, which allow euryhaline fishes to have sensitive perception to the environmental osmotic conditions (Kültz 2012, 2015). Euryhaline fishes have intricate physiologies, and genomic approaches may help us to identify broad genomic patterns across populations of euryhaline fish (Barrett and Hoekstra 2011).

Evolutionary theory suggests that high levels of phenotypic plasticity may lower the potential for local adaptation and speciation (Snell-Rood et al. 2010, Thibert-Plante and Hendry 2011). Hence, an expectation for fish species with high levels of salinity tolerance is that

isolation by distance would predominate. On the other hand, plasticity may be sufficient for organisms to tolerate a wide variety of different salinities, but that subsequent local adaptation in different environments alters allele frequencies at functionally important loci and ultimately leads to a pattern if isolation by adaptation.

The Fundulidae family (North American killifish) is composed of various fresh, brackish, and marine environments and is a model system for studying the effects of dispersal and local adaptation on population structure (Griffith 1974, Wood and Marshall 1994, Whitehead 2010). Within this family, multiple marine to fresh water transitions have occurred independently (Fuller and Noa 2008, Whitehead 2010). Previous work has suggested that adaptive divergence along salinity gradients is present in this group. In *Fundulus heteroclitus*, genetic and physiological approaches comparing freshwater and saltwater populations have indicated population divergence consistent with local adaptation as a function of salinity (Brennan et al. 2018): Freshwater and saltwater populations are genetically distinct as a function of salinity and have salinity-dependent physiological differences (Duvernell et al. 2008, Whitehead et al. 2011, Ghedotti and Davis 2013, Brennan et al. 2018). Closely related to *F. heteroclitus* are the fundulids *Lucania parva* and *L. goodei,* notable for being closely related yet having a large difference in their salinity tolerance (Wood and Marshall 1994, Fuller et al. 2007, Whitehead 2010). The euryhaline species *L. parva* is particularly remarkable for its broad salinity tolerance, and has established permanent populations in fresh, brackish, and salt water ranging from Cape Cod to the Atlantic and Gulf coasts (Hubbs and Miller 1965, Page and Burr 1991). As discussed in chapter 1, there is evidence suggesting salinity has dramatic effects on the biology of *Lucania* species. Notably, Kozak et al. (2014) used pooled transcriptome data and GO analyses to compare gene expression in *Lucania* and found highly differentiated SNPs for ion transport

genes and osmoregulatory genes not only between *L. parva* and *L. goodei*, but also between freshwater and saltwater populations of *L. parva*. The analysis also implicated differentiation in tight junction genes between freshwater and saltwater populations of *L. parva*, which is interesting because tight junction expression is often upregulated in freshwater to prevent the loss of ions (Tipsmark and Madsen 2012, Kozak et al. 2014).

This approach of using transcriptome data somewhat limits the results to genes expressed in the gills, ovaries, and testes. Given the complexity of osmoregulation by euryhaline fishes such as *L. parva*, it is possible that there is more standing variation that is associated with divergence in osmotic environments, which would be consistent with isolation by environment. Conversely, variation in the genome may be more easily explained by isolation by distance if populations in different drainage systems have higher levels of F_{ST} than populations within the same drainage system. With this scenario, limited gene flow would lead to genetic differentiation. In order to understand how euryhalinity whether populations of *L. parva* are locally adapted to their native salinity, it is important to understand what may drive differentiation between various *L. parva* populations.

In this study, I use restriction site-associated DNA sequencing (RAD-Seq) for 10 populations of *L. parva* from freshwater, brackish, and marine populations across Florida in order to examine the effects of salinity and distance on population structure. The goal of this study was to examine population structure in *L. parva* and to determine whether salinity habitat results in higher differentiation than expected as a function of river drainage and distance.

METHODS AND MATERIALS

Field collection

In June 2017, rainwater killifish, *L. parva*, were collected from ten sites across Florida (Figure 3.2, Table 3.1). Fish were collected with dipnets and seines and held in aerated coolers until they were euthanized with an overdose of MS-222. Each individual was sexed and photographed. We placed a small cut in the abdomen of the fish and subsequently stored the animal in 95% ethanol. The samples were returned to the University of Illinois and stored at -20C until DNA extraction at a later time. Sample sizes were as follows: St. John's/Atlantic drainage system populations Palatka n=20, Delks Bluff n=17, and Merritt Island n=20; Everglades/Keys populations Alabama Jack's n=16 and Key West n=20; Suwannee populations Mouth of Suwanee n=3 and California Creek n=19; Wakulla populations Lighthouse Pond n=20 and Lower Bridge n=20; and Panhandle population St. George n=20.

RAD-Seq and population genetics analysis

We performed single-digest RAD-Seq to investigate population structure. DNA was extracted from the skin and muscle tissue from 175 individuals using a Qiagen DNeasy Blood & Tissue kit (96-well plate) and treated with RNase A. DNA concentrations were quantified using a Qubit fluorometer and normalized to a concentration of 20 ng/uL in 50 uL 1x TE. A RADseq library was prepared with the restriction enzyme SbfI by Floragenex (Eugene, OR, USA), following the methods of Baird et al. (2008). The 175 individuals from this study were duplexed with 205 individuals from another study for a total of 380 samples. RAD libraries were sequenced as single-end 100 bp reads on two lanes in an Illumina HiSeq 4000 machine.

Sequencing resulted in a total of 311,522,855 reads across the 175 individuals, with a mean ± SE of 1,780,131 ± 793,957 reads per individual. We used the *process_radtags* program in Stacks version 1.48 (Catchen et al. 2011, 2013) to demultiplex samples, remove barcodes, and remove reads of low quality or with ambiguous barcodes. This resulted in a total of 305,663,891 retained reads, which were supplied to the *denovo_map* pipeline in Stacks to construct a catalog of loci and called SNPs. These were anonymous SNPs that were not aligned to a genome or a transcriptome.

For each locus, a minimum of three identical reads were required (-m 3), with a maximum of three mismatches between loci in each individual (-M 3), and a maximum of two mismatches between loci to be added to the catalog (-n 2). This resulted in a catalog of 854,545 variant sites across 21,821,757 loci, and a mean ± SE depth of coverage of 21.8 ± 6.5 per individual.

The *populations* program in Stacks was used to generate population genetic statistics. We required that loci were present in all ten populations (-p 10) and in at least 60% of the individuals within a group (-r 0.6), with a minimum minor allele frequency of 3% (--min_maf 0.03). After filtering, a total of 1,268,756 total sites across the genome were retained. There were 28,839 variant sites (i.e., contained a SNP) across 13,597 RAD loci. To measure the level of genetic diversity within groups, we used *populations* to obtain estimates of nucleotide diversity (π), heterozygosity, the percent of polymorphic sites, and the number of private alleles. We used *populations* to generate statistics of genetic differentiation between groups, including SNP-based AMOVA F_{ST} (Weir 1996), haplotype-based Φ_{ST} (Excoffier et al. 1992), and D_{EST} (Jost 2008).

We tested the effects of distance, river drainage, east-west divide, and salinity on population structure (i.e., F_{ST}) using mantel and partial mantel tests of matrix similarity. Figure

2.1 shows the location of drainages and populations used in this study. The mantel test is essentially a correlation of two matrices. The partial mantel test is a partial correlation between two matrices after controlling for a third. We used the F_{ST} matrix among all populations as an estimate of genetic differentiation. For the distance matrix, we calculated the distance between populations via water. For some population pairs, this was the distance from north Florida populations on the east side of the state around the peninsula to north Florida populations on the west side of the state. For the drainage matrix, we coded populations as '0' if they occurred in the same drainage and '1" if they occurred in different drainages. We also made an 'east-west' drainage matrix where three populations from the St. John's drainages were in the 'east' and the North Florida (St. George Island, Lower Bridge of the Wakulla River, and Lighthouse Pond), Suwannee (Mouth of Suwanee, California Creek), and Everglades/Keys (Alabama Jack's, Key West) populations were the 'west'. For the salinity matrix, we calculated the absolute difference in salinity between the populations. We first considered the mantel correlation between the F_{ST} matrix and three other matrices (distance, river drainage, east-west drainage, and salinity). We then asked whether there was a significant relationship between F_{ST} and salinity after controlling for distance, river drainage, and east-west drainage using a partial mantel test. For all tests, statistical significance was assessed with 5,000 permutations of the matrices. The analyses were conducted in R using the 'vegan' package (Oksanen et al. 2019).

We also performed a principal components analysis on the RAD-Seq data and to ask whether the component scores differed as a function of river drainage or salinity. If the individuals clustered with other individuals from the same river drainage system regardless of salinity, then that would be evidence of distance being a better predictor of genetic differentiation in *L. parva*. If populations of from similar salinities and different drainage

systems clustered together then that would suggest that there are consistent genetic changes in the genome as functions of salinity. We used a GenePop file generated from *populations* containing genotype data for all 28,839 SNPs for a principal component analysis (PCA) in 'adegenet' in R (version 2.1.2; Jombart 2008). In order to measure the strength of linear association between the population means of axes and population salinities, Pearson's product-moment correlation was measured. Finally, we performed ANOVA tests on the PCA axis values to determine if the genetic diversity among populations was due to drainage or salinity.

RESULTS

F_{ST} and population genetics statistics

The genetic diversity within each of the 10 *L. parva* populations was generally low (Table 2.2). The nucleotide diversity (π) was about 0.003 across all the populations. The observed heterozygosity was similarly low across all populations, and slightly lower than the expected heterozygosity. There was more variation in the percentage of polymorphic sites across all loci and in the number of private alleles in each population (Table 2.2). The number of private alleles was highest in the St. George's Island population (4.4X higher than the average of 164.8 private alleles per population) and in the Merritt Island population (2.7X higher than average). Interestingly, the Wakulla (Lower Bridge-Lighthouse Pond) and Suwanee (Mouth of Suwanee-California Creek) population pairs had few private alleles, which is an indicator of gene flow or recent shared ancestry/more recent divergence between populations.

Comparisons between populations also yielded generally low genetic differentiation, where the highest measure being only 0.287 Φ_{ST} between Key West and Delks Bluff (Tables 2.3A-C). The SNP-based F_{ST} was on average 0.058 lower compared to the haplotype-based Φ_{ST}

and 0.040 lower than the D_{EST}, but all measures showed similar patterns of genetic differentiation (Tables 2.3A-C). Notably, genetic differentiation within the western river drainages (Wakulla, St. George's and Suwanee Drainage systems) were lowest (F_{ST} from 0.0270-0.0875, Φ_{ST} from 0.011-0.1715, and D_{EST} from 0.0075-0.1461). The genetic differentiation between Delks Bluff and Merritt Island, a freshwater-saltwater pair in the St. John's drainage system, was high in comparison to the western drainages (F_{ST} = 0.1857, Φ_{ST} = 0.2782, and D_{EST} = 0.2579).

Similar patterns can be seen in Figure 2.2, which shows the F_{ST} distributions between different population pairs. The smallest mean F_{ST} values were within the western river drainages (Suwanee and Wakulla, Figure 2.2C-D). The mean F_{ST} values and variability in F_{ST} at individual loci were greater within the St. John's and Everglades/Keys drainages when comparing populations from similar salinities (Figure 2.2E-F). The mean F_{ST} scores and largest variation in F_{st} values at individual loci were between the freshwater-saltwater populations in the St. John's drainage system (Figure 2.2A-B). The St. John's drainage also has a greater waterway distance between the freshwater and saltwater sampled populations.

Mantel Analysis

There was a very strong correlation between F_{ST} and east-west drainage (r = 0.58, p = 0.0084) and a moderately strong correlation between F_{ST} and river drainage (r = 0.22, p = 0.024). The relationship between F_{ST} and salinity was positive (r = 0.24) but did not rise to the level of statistical significance (p = 0.1116). A partial mantel test did reveal a significant, positive relationship between F_{ST} and salinity when east-west drainage was controlled for (r = 0.398, p = 0.019).

Principal Component Analysis

The PCA analysis of 28,839 SNPs across the 175 individuals also showed strong effects of distance on population structure, with some effects of salinity and their interaction as well. The PCA generated 174 possible dimensions, of which the first five, which accounted for the most variation in the data (8.076 to 1.693%, for a total of 25.49%) were further analyzed. The ANOVA tests on PCA Axes 1-4 indicated strong effects of drainage (Table 2.4). The effect of salinity was also significant for all five PCA axes. However, only Axis 3 had a significant Pearson's product-moment correlation for salinity (r=-0.6792, t=-2.6173, df=8, p=0.0308, Table 2.5). For PC3, populations of similar salinities clustered together. Axis 1 accounted for 8.71% of the variation in SNPs and was largely attributable to differences between the eastern and western drainages. Axis 2 accounted for 6.26% of the variation and was attributable to differences in salinity in the eastern, but not western, drainage. Axis 3 accounted for 5.59% of the variation and was attributable to differences between the Panhandle and the Everglades/Keys drainages. This is consistent with the mantel analyses which revealed a strong signature of river drainage and east-west drainage and a significant, but a weaker, signature of salinity.

DISCUSSION

In this study, I tested the hypothesis that differences in salinity drive genetic differentiation in the euryhaline fish *L. parva* beyond what would be expected from distance alone. Using RAD-Seq data from 10 populations, I found strong evidence for effects of drainage where populations in the same drainage system had lower measures of genetic differentiation, and some evidence for effects of salinity and the interaction between distance and salinity. These results are consistent with the idea that *L. parva* has high dispersal along the coast through

marine and brackish water populations with multiple, independent invasions of inland, freshwater sites. The significant, but weaker, effect of salinity is consistent with the idea that, while broadly tolerant of a wide range of salinities, that different salinities may favor different alleles in fresh versus salt water.

I found generally low genetic differentiation among populations of *L. parva*. Previous studies have also shown similar patterns of low to moderate genetic differentiation between populations of *L. parva* (Duggins et al. 1983, Kozak et al. 2014). Interestingly, this analysis showed particularly low levels of genetic divergence within the North Florida and Suwanee population pairs. This and the finding that the freshwater populations in these drainages had no private alleles strongly suggest multiple freshwater invasions across Florida by nearby saltwater populations rather than a single invasion and dispersal into fresh water. Even when barriers such as land separate formerly connected populations, the genetic structure of the populations may reflect historic connectivity to other populations (Poissant et al. 2005). Alternatively, this may also indicate that these populations are recently diverged, or there is still gene flow in these drainages. The St. John's populations, in comparison, showed much higher divergence, especially between the saltwater population and the two freshwater populations. It is also noteworthy that St. John's freshwater populations were further inland and there is more physical distance between these populations. This may also implicate isolation by distance within this group. The PCA analysis of 28,839 SNPs again identified drainage as having significantly high effect on axes 1-4, accounting for a combined total of 23.8% of the variation.

The findings that distance has strong effects on population structure in this system is not wholly surprising. Many studies in fish have implicated isolation by distance as an important factor contributing to population structure (Chenoweth et al. 1998, Olden et al. 2001, Bradbury

and Bentzen 2007, Pinsky et al. 2010, Crookes and Shaw 2016). In a study by Adams et al. 2006 on *F. heteroclitus*, patterns of isolation by distance were observed both within and between northern and southern regions, and further analysis indicated geographical distance accounted for 78.9% of the variance in genetic diversity.

Though I found strong effects of isolation by distance, I did find some evidence that suggests effects of salinity on population structure in *L. parva*. Across populations, here was a significant correlation between F_{ST} and salinity differences when the effects of east-west drainage were accounted for. Also, when comparing the two saltwater populations in the South Florida drainage system to all the other populations, we can see that population-wide F_{ST} was generally more elevated when compared to freshwater populations than saltwater populations (with the notable exception of Lower Bridge, which appears to be genetically similar to the nearby saltwater population, Table 2.3A). Our PCA analysis also seems to indicate that there is some effect of salinity on these populations. Though we did not investigate which SNPs are contributing to the elevated F_{ST} scores or to specific PCA axes, it is possible that some of these SNPs are occurring in osmoregulatory genes, as was suggested by Kozak et al. (2014). Similar patterns of divergent loci between freshwater-saltwater populations has also been found in threespine sticklebacks (*Gasterosteus aceuleatus*; Hohenlohe et al. 2010, Jones et al. 2012) and in *F. heteroclitus* (Brennan et al. 2018).

Of course, *L. parva* are extremely tolerant of a wide range in salinities, and this extreme euryhalinity has been preserved even in far inland freshwater populations. According to theory, the ability to be perfectly plastic will decrease the strength of natural selection because plasticity will mask differences among genotypes (Thompson 1991). Hence, the expectation is that plasticity reduces the scope for local adaptation as a function of salinity. The fact that salinity has

effects on F_{ST} and PC scores suggests that there is scope for natural selection to affect allele frequencies. In addition, salinity affects many aspects of the environment beyond simple ion concentrations. Salinity has strong effects on community structure which can alter predator/prey relationships, resources for consumption, and the outcome of competition. In chapter 3, I directly test whether there is evidence for local adaptation between a freshwater (Lower Bridge) and a saltwater (Lighthouse Pond) population using a laboratory experiment where I raised offspring from the two populations under different salinities and measured survival.

TABLES AND FIGURES

Table 2.1. Collection site information. Salinities less than 1 ppt were considered fresh, 1-10 ppt were considered intermediate, and above 10 ppt (the isosmotic point) were considered saltwater.

Site	Drainage	County	Site type	Salinity (ppt)
Palatka	St. John's/ Florida Atlantic	Putnam	Fresh	0.8
Delks Bluff	St. John's/ Florida Atlantic	Marion	Fresh	0.2
Merritt Island	St. John's/ Florida Atlantic	Brevard	Saltwater	21.5
Alabama Jack's	Everglades/Keys	Miami-Dade	Saltwater (Marine)	34.5
Key West	Everglades/Keys	Monroe	Saltwater (Marine)	34
Mouth of Suwanee	Suwanee/ Big Bend	Dixie	Fresh	0.2
California Creek	Suwanee/ Big Bend	Dixie	Intermediate	5.0
Lighthouse Pond	Wakulla/ North Florida	Wakulla	Saltwater	18.2
Lower Bridge	Wakulla/ North Florida	Wakulla	Fresh	0.3
St. George Island	Panhandle/North Florida	Franklin	Fresh	0.8

Table 2.2. Measurements of genetic diversity within populations across all 1,268,756 loci. % Poly = percent polymorphic sites, # Private = number of private alleles, π = nucleotide diversity, H_{OBS} = observed heterozygosity, H_{EXP} = expected heterozygosity.

Population	**n**	**% Poly**	**# Private**	**π**	**H_{OBS}**	**H_{EXP}**
Palatka	20	1.3320	67	0.0036	0.0027	0.0035
Delks Bluff	17	0.9787	212	0.0032	0.0023	0.0031
Merritt Island	20	0.9434	443	0.0029	0.0020	0.0028
Alabama Jack's	16	1.1376	18	0.0032	0.0016	0.0031
Key West	20	0.9613	164	0.0031	0.0013	0.0021
Mouth of Suwanee	3	0.5386	0	0.0025	0.0015	0.0021
California Creek	19	1.2787	1	0.0029	0.0020	0.0028
Lighthouse Pond	20	1.2503	15	0.0029	0.0021	0.0029
Lower Bridge	20	1.1835	0	0.0029	0.0020	0.0028
St. George	20	0.9383	728	0.0029	0.0023	0.0028

Table 2.3A. SNP-based fixation statistic (F_{ST}) between populations. F_{ST} was calculated using 28,839 variant sites (SNPs). Freshwater-saltwater drainage comparisons are in bold. Darker boxes indicate higher F_{ST} values, grouped as: ≥0.050, 0.051-0.100, 0.101-0.150, and ≤0.151.

	St. John's/Florida Atlantic			Everglades/Keys		Suwanee/Big Bend		Wakulla/North Florida		Panhandle
	Palatka	Delks Bluff	Merritt Island	Alabama Jack's	Key West	Mouth of Suwanee	California Creek	Lighthouse Pond	Lower Bridge	St. George
Palatka	X	0.0657	**0.1144**	0.1040	0.1325	0.0799	0.0802	0.0819	0.0870	0.1285
Delks Bluff		X	**0.1857**	0.1539	0.1878	0.1455	0.1247	0.1296	0.1311	0.1831
Merritt Island			X	0.1487	0.1836	0.1386	0.1191	0.1239	0.1260	0.1806
Alabama Jack's				X	0.0615	0.0808	0.0659	0.0690	0.0708	0.1207
Key West					X	0.1128	0.0963	0.0998	0.1018	0.1568
Mouth of Suwanee						X	**0.0392**	0.0476	0.0395	0.0597
California Creek							X	0.0244	0.0270	0.0800
Lighthouse Pond								X	**0.0290**	0.0875
Lower Bridge									X	0.0836
St. George										X

Table 2.3B. Haplotype-based Φ_{ST} between populations. Φ_{ST} was calculated using 1,268,756 sites across 13,597 loci. Freshwater-saltwater drainage comparisons are in bold. Darker boxes indicate higher Φ_{ST} values, grouped as: ≥0.070, 0.071-0.140, 0.141-0.210, and ≤0.211.

	St. John's/Florida Atlantic			Everglades/Keys		Suwanee/Big Bend		Wakulla/North Florida		Panhandle
	Palatka	Delks Bluff	Merritt Island	Alabama Jack's	Key West	Mouth of Suwanee	California Creek	Lighthouse Pond	Lower Bridge	St. George
Palatka	X	0.0966	**0.1784**	0.1683	0.2151	0.1367	0.1287	0.1417	0.1306	0.2098
Delks Bluff		X	**0.2782**	0.2374	0.2867	0.2476	0.1967	0.2102	0.2027	0.2826
Merritt Island			X	0.2348	0.2827	0.2569	0.1921	0.2032	0.1999	0.2810
Alabama Jack's				X	0.0822	0.1080	0.0946	0.1082	0.0960	0.1928
Key West					X	0.1972	0.1517	0.1637	0.1566	0.2459
Mouth of Suwanee						X	**0.0169**	0.0344	0.0235	0.1715
California Creek							X	0.0249	0.0111	0.1249
Lighthouse Pond								X	**0.0257**	0.1384
Lower Bridge									X	0.1300
St. George										X

Table 2.3C. Haplotype-based D_{EST} between populations. D_{EST} was calculated using 1,268,756 sites across 13,597 loci. Freshwater-saltwater drainage comparisons are in bold. Darker boxes indicate higher D_{EST} values, grouped as: ≥0.070, 0.071-0.140, 0.141-0.210, and ≤0.211.

	St. John's/Florida Atlantic			Everglades/Keys		Suwanee/Big Bend		Wakulla/North Florida		Panhandle
	Palatka	Delks Bluff	Merritt Island	Alabama Jack's	Key West	Mouth of Suwanee	California Creek	Lighthouse Pond	Lower Bridge	St. George
Palatka	X	0.0856	**0.1607**	0.1419	0.1897	0.1397	0.1064	0.1147	0.1103	0.1797
Delks Bluff		X	**0.2579**	0.2128	0.2632	0.2286	0.1760	0.1847	0.1824	0.2546
Merritt Island			X	0.2094	0.2610	0.2267	0.1716	0.1806	0.1783	0.2539
Alabama Jack's				X	0.0718	0.1085	0.0755	0.0838	0.0786	0.1611
Key West					X	0.1763	0.1312	0.1384	0.1359	0.2163
Mouth of Suwanee						X	**0.0316**	0.0424	0.0336	0.1461
California Creek							X	0.0174	0.0075	0.1035
Lighthouse Pond								X	**0.0180**	0.1142
Lower Bridge									X	0.1079
St. George										X

Table 2.4. ANOVA results for PCA Axes, drainage systems, and salinity.

PCA Axis	Response	Sum Sq.	df	F	p
1	Intercept	2499	1	14.517	**<0.0005**
	Drainage	777,901	4	1129.773	**< 0.0005**
	Salinity	12,375	1	71.893	**< 0.0005**
	Residuals	29,091	169		
2	Intercept	270,690	1	181.667	**< 0.0005**
	Drainage	251,831	4	42.252	**< 0.0005**
	Salinity	359,075	1	240.984	**< 0.0005**
	Residuals	251,816	169		
3	Intercept	12	1	0.0844	0.7717
	Drainage	277,216	4	491.5682	**< 0.0005**
	Salinity	5,588	1	39.6357	**<0.0005**
	Residuals	23,827	169		
4	Intercept	2,210	1	7.3519	**0.0074**
	Drainage	261,090	4	217.1763	**< 0.0005**
	Salinity	1,759	1	5.8514	**0.0166**
	Residuals	50,793	169		
5	Intercept	5,510	1	5.8342	**0.0167**
	Drainage	8,529	4	2.2580	0.0649
	Salinity	9,036	1	9.5681	**0.0023**
	Residuals	159,595	169		

Table 2.5. Pearson's product-moment correlations between salinity and population-average Axis scores.

Axis	r	t	df	p
1	-0.2643	-0.07751	8	0.4606
2	-0.4375	-1.3763	8	0.2060
3	**-0.6792**	**-2.6173**	**8**	**0.0308**
4	-0.6792	0.8646	8	0.4124
5	-0.0810	-0.2299	8	0.8239

Figure 2.1. *L. parva* populations and drainage systems used in this study: 1=Palatka, 2=Delks Bluff, 3=Merritt Island, 4=Alabama Jack's, 5=Key West, 6=Mouth of Suwanee, 7=California Creek, 8=Lighthouse Pond, 9=Lower Bridge, 10=St. George's Island.

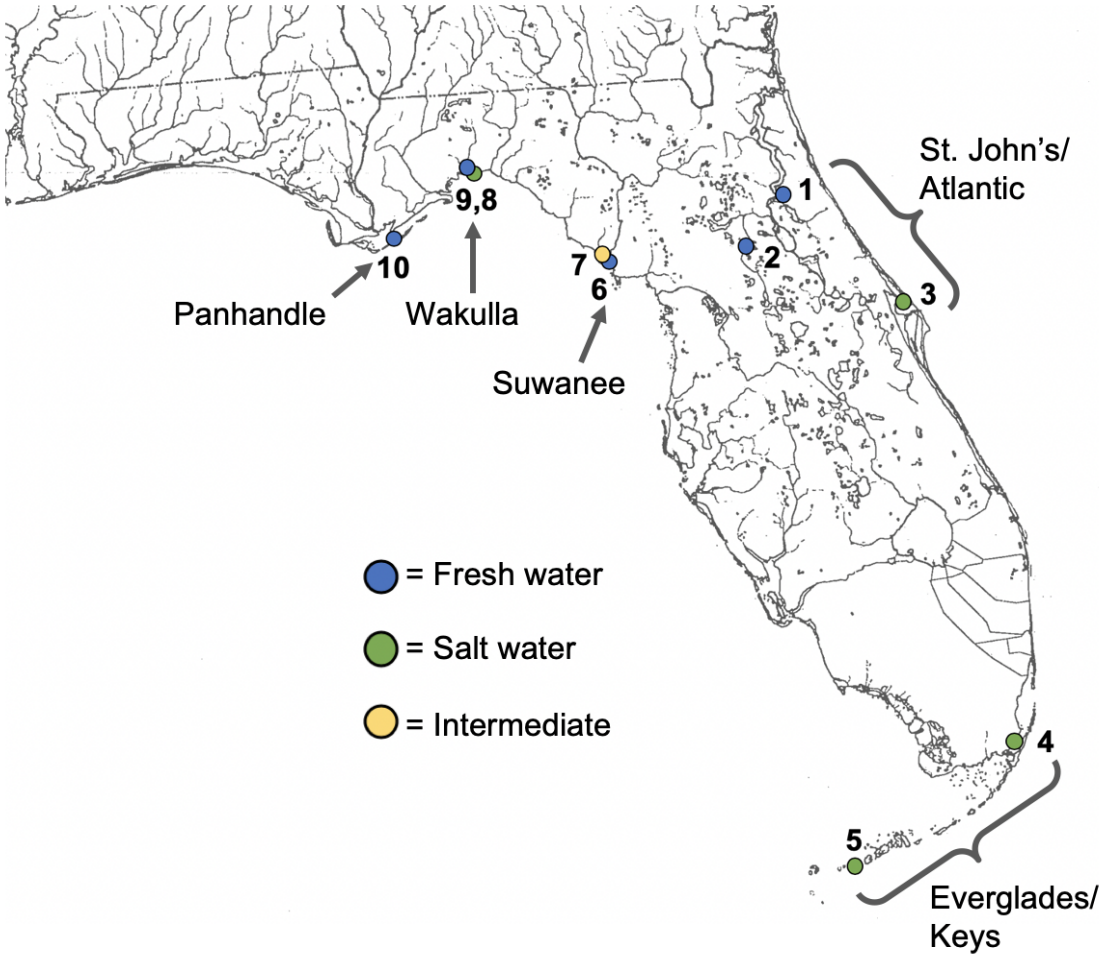


Figure 2.2. AMOVA F_{ST} distribution between freshwater-saltwater population pairs from (A) St. John's/Atlantic (Palatka vs Merritt Island), (B) St. John's/Atlantic (Delks Bluff vs Merritt Island), (C) Suwanee/Big Bend, and (D) Wakulla/North Florida; between freshwater populations from (E) St. John's/Atlantic (Palatka vs Delks Bluff); and between saltwater populations from (F) Everglades/Keys.

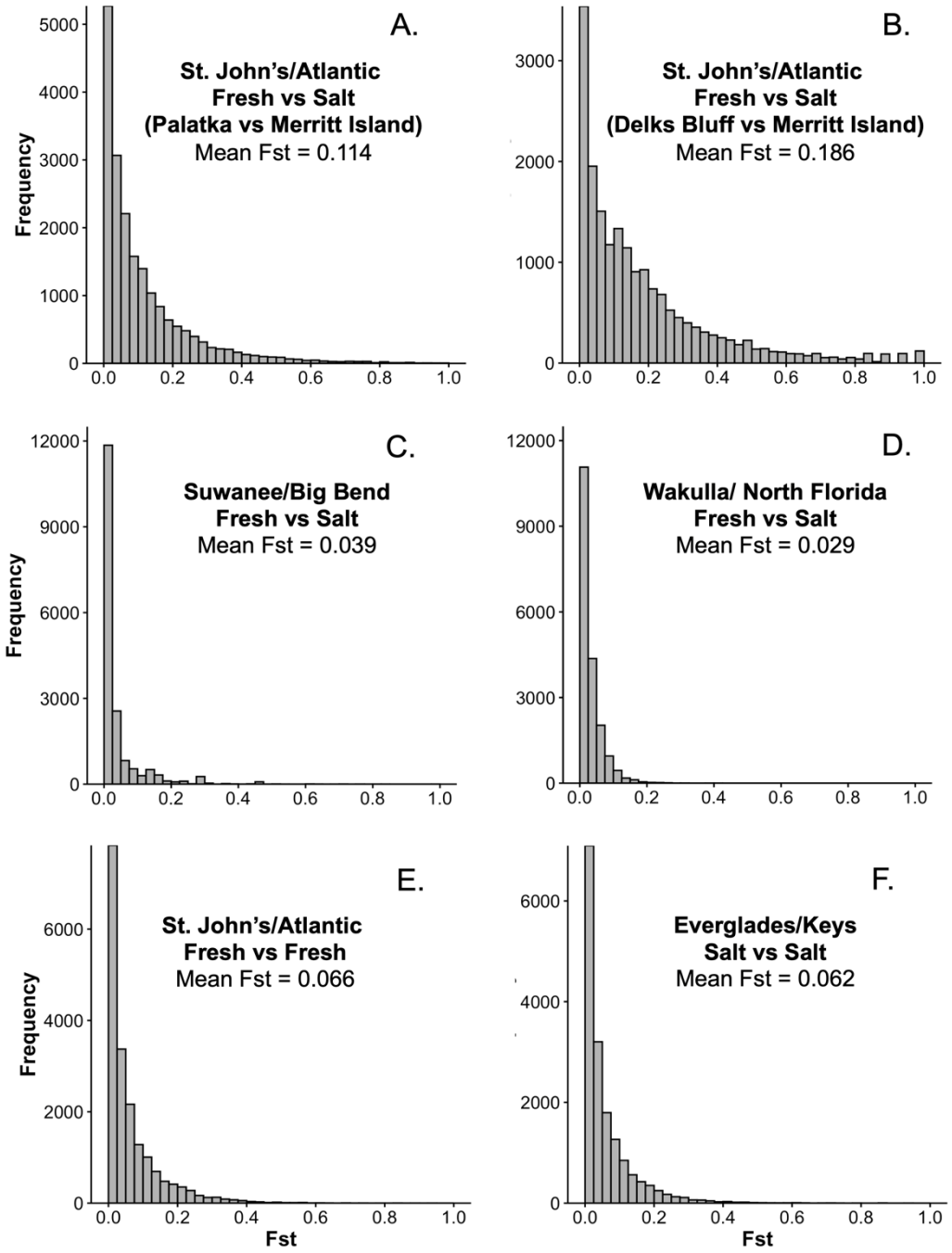
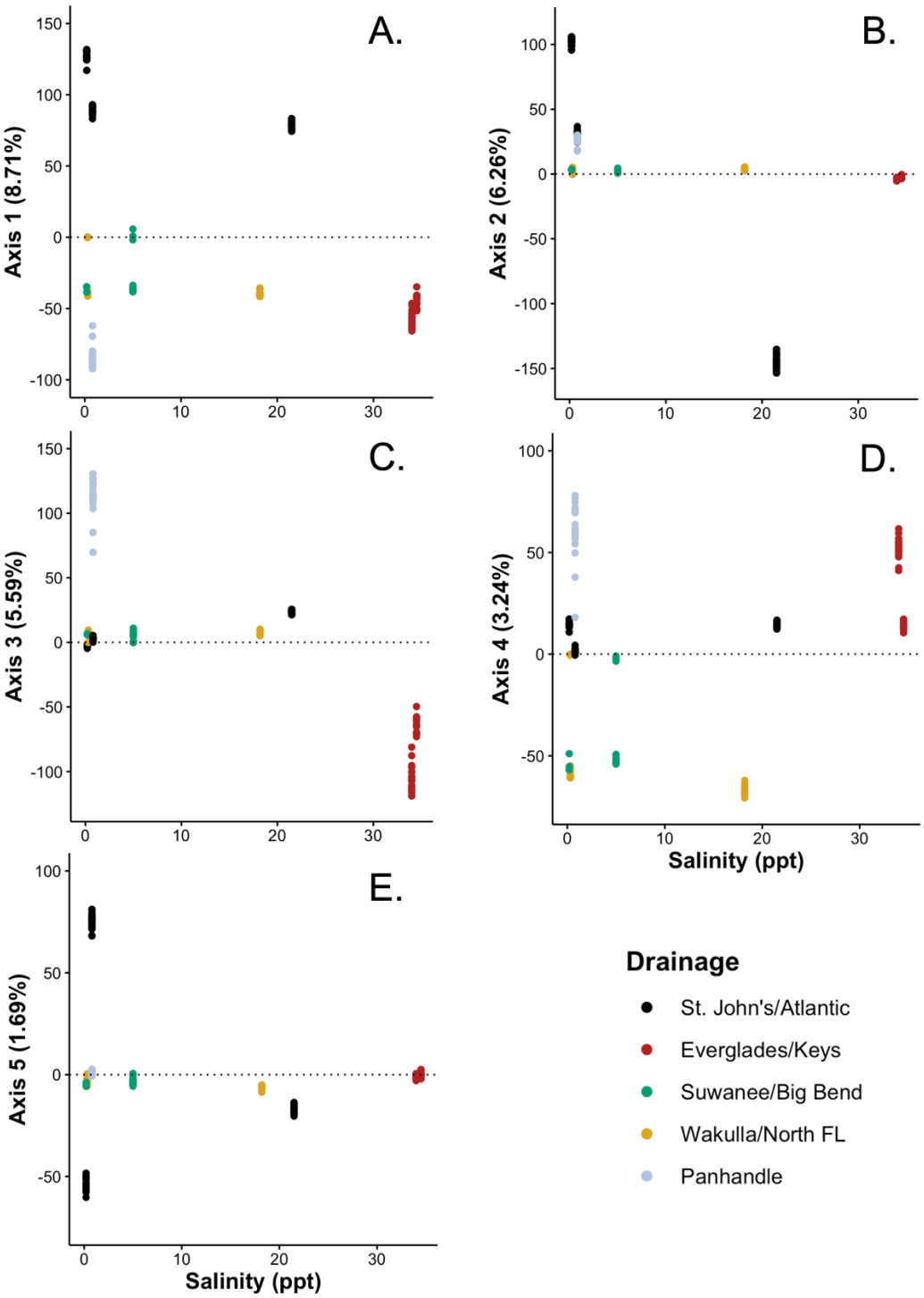


Figure 2.3. Plots of PCA Axes against salinities of collection sites, by drainage. (A) Axis 1 (B) Axis 2, (C) Axis 3, (D) Axis 4, (E) Axis 5.



CHAPTER 3: A TEST FOR LOCAL ADAPTATION AND ADAPTIVE MATERNAL EFFECTS IN *LUCANIA PARVA*

ABSTRACT

In over 95% of fish species, eggs develop outside the body of the parent. The consequence is that eggs and the resulting larval fish undergo development while facing the demands of maintaining homeostasis in the external environment. In some euryhaline species, this means that eggs/embryos might face the possibility of growing and developing under fresh, brackish, or marine conditions. This is astonishing given that fresh and salt water create opposing demands on ion/osmoregulation. The evolutionary mechanisms that allow for development under such diverse conditions in a single species are unclear. One possibility is that individuals simply possess ion/osmoregulation mechanisms for both freshwater and salter osmoregulation. However, maintaining both the physiological freshwater and saltwater osmoregulatory mechanisms would seem costly as it requires the maintenance of both the freshwater and saltwater active transport ion pumps in the gill. Another possibility is that there is local adaptation to freshwater and saltwater conditions. Yet another possibility is that mothers alter the properties/contents of the egg as a function of the salinity environment in which they currently occur. Maternal effects might be particularly important in the early stages of development when fish lack gills and other organs important for ion/osmoregulation. In this study, I examined the extent of local adaptation and maternal effects as a function of salinity between freshwater and saltwater populations of the rainwater killifish, *Lucania parva*. I sampled fish from two populations in North Florida – one found consistently in fresh water (0.2 ppt) and another found consistently in salty brackish water (20 ppt). I performed crosses in low and high salinities and

raised offspring under various salinities. I found strong evidence that the salinity in which offspring were raised had large effects on offspring survival, but the pattern was not consistent with local adaptation. There was also evidence for maternal effects, but these did not appear to be adaptive (i.e., parents that spawned in fresh water did not increase the survival of offspring raised in freshwater). There was also an interaction between population and spawning salinity where offspring from freshwater parents who spawned in fresh water had increased survival from day 5 to hatching.

INTRODUCTION

Ion and osmo-regulation (the active regulation of internal ion and water levels at particular levels) is critical in vertebrates to preserve cellular, physiological, and neural processes (Loretz and Bern 1982, Hwang et al. 2011, Kültz 2015). Aquatic organisms face particular challenges because - in the lack of active processes - passive diffusion and osmosis result in internal ion and water levels converging on those of the external environment (Parry 1966, Evans and Claiborne 2008). Most fish species can only tolerate either a freshwater or a saltwater environment, but cannot switch between the two due to the opposing osmotic needs and stresses fresh water and salt water environments impose (Gunter 1945, 1950, Whitfield 2015, Nelson et al. 2016). Teleost fishes regulate their plasma osmotic concentration to be about one-third that of sea water; thus, fishes in fresh water must compensate for the passive gain of water and loss of ions to the environment by producing high quantities of diluted urine and minimizing renal salt loss, while fishes in salt water must compensate for the passive loss of water and gain of ions to the environment by ingesting salt water and secreting ions out the gills (McCormick 2001, Evans et al. 2005). The physiological mechanisms involved in osmoregulation vary greatly between

fresh water and marine fishes (reviewed in Evans et al. 2005). Notable differences include hormone production that stimulates the salinity-appropriate behaviors and the alternate forms and functions mitochondria-rich cells in freshwater and saltwater fish (i.e. producing H^+-ATPase in fresh water and Na^+/K^+-ATPase in salt water; Evans et al. 2005). In stable environments, most fish species are stenohaline, specializing in either fresh water or salt water (Kültz 2015).

Euryhaline species are of note because of their ability to tolerate a broader range in salinity and represent a small subset of fish species (Whitfield 2015, Kültz 2015, Nelson et al. 2016). Many euryhaline species like salmon or eels are diadromous, meaning they migrate between fresh and saltwater environments (McDowall 1997, Zydlewski and Wilkie 2012). Anadromous species, such as salmon, are spawned in fresh water, then migrate to marine environments before returning to fresh water as adults to breed (McDowall 1997, Björnsson et al. 2011, Nelson et al. 2016). Catadromous species, such as European and North American eels, do the reverse, being spawned in the sea, migrating to fresh water, then returning to the sea to breed (McDowall 1997, Nelson et al. 2016, Cao et al. 2018). In both anadromous and catadromous species, the transition between freshwater and saltwater environments occurs at predictable times during specific life history stages (McDowall 1997, Björnsson et al. 2011, Zydlewski and Wilkie 2012). These fishes typically do not transition rapidly from one salinity habitat to another. Instead, they typically spend prolonged periods of time at the coasts as they undergo elaborate changes to their osmoregulatory system (Björnsson et al. 2011, Zydlewski and Wilkie 2012).

In contrast, other euryhaline species, such as killifish, typically live in environments with less predictable salinity levels (Whitehead 2010, Marshall 2012, Whitfield 2015, Kültz 2015). Generations may be spent in just fresh water or salt water, but there may be times when the salinity changes rapidly. Extreme weather events can cause a sudden and significant changes in

salinity (Cardoso et al. 2008, Kültz 2015). Extreme rain and floods can decrease salinity. Hurricanes can lead to sudden increases in salinity as storm surges push sea water into inland waters (Walker 2001, Illangasekare et al. 2006, Fuller 2008a). Droughts can also increase salinity as evaporation of water increases the concentration of salt (Nielsen and Brock 2009). These fish have to possess the physiological mechanisms to rapidly accommodate fresh, brackish, or marine habitats, including osmo- and ionoreceptors that can stimulate changes in the gill epithelium (Wood and Marshall 1994, Evans et al. 2005, Kültz 2015).

Maintaining these generalist mechanisms requires that species possess both freshwater and saltwater osmoregulatory mechanisms (Scott and Schulte 2005, Berdan and Fuller 2012b). This likely comes at a significant cost, which is why euryhalinity is not widespread (Hwang et al. 2011, Schultz and McCormick 2012, Wrange et al. 2014). In more stable environments, adaptions for a specific salinity environment may be favored, and deleterious mutations affecting osmoregulation in other salinities may go unpurged (Scott and Schulte 2005, Schulte 2007, Schultz and McCormick 2012). Osmoregulation during early life stages is also likely to be influenced by significant maternal effects. The embryo itself is not capable of active, adult-like osmoregulation until shortly after hatching, when the gill filaments and other tissues mature (Guggino 1980, Alderdice 1988, Varsamos et al. 2005). Basic osmoregulation in killifish begins around days 3-4 of development, when embryonic ionocytes like chloride cells develop on the yolk sac epithelium of the embryo (Guggino 1980). Hence, osmoregulation in the early egg stage involves a combination of maternal effects (e.g., the properties of the egg shell itself, potential RNAs/proteins from the mother, and mitochondria-rich cells), water-permissiveness of plasma membranes, and embryonic ionocytes (Alderdice 1988). The extent to which mothers alter these as a function of the environment is unknown. Adaptive maternal effects would result in increased

offspring survival in environments where the maternal environment is an indicator of the environment the offspring will experience (Ezard et al. 2014)

Study System

In this study, I investigate the extent to which genetic and environmental effects influence salinity tolerance in killifish. The rainwater killifish, *Lucania parva*, is a euryhaline species of particular interest because of its remarkable tolerance in a wide range in salinities (Hubbs and Miller 1965). There are permanent populations of *L. parva* in fresh, brackish, and salt water ranging from Cape Cod to the Atlantic and Gulf coasts (Hubbs and Miller 1965, Page and Burr 1991). Freshwater *L. parva* have derived from brackish and marine populations independently multiple times (Fuller and Noa 2008). Previous studies indicate that *L. parva* has higher fitness in brackish and salt water (Fuller et al. 2007, Fuller 2008a, 2008b). Though *L. parva* appears to have high hatching success in fresh water, overwinter survival to adulthood declines compared to fish raised in salt water (Fuller et al. 2007, Fuller 2008b, Fuller and Noa 2008). There is some evidence that suggest *L. parva* populations may have local adaptations as a function of salinity.

Kozak et al. (2014) conducted a two-pronged study that examined gene expression via microarrays and that examined population structure by examining SNPs from transcriptomes. The transcriptomes were created for six populations of *L. parva* and two populations of the sister species, *L. goodei.* A reference transcriptome was created using the combined transcriptomes of all 6 populations, and then each populations was aligned separately. Then, SNP detection and F_{ST} calculations were be done using the pooled population data, allowing for comparisons of outlier loci between the populations. The transcriptome comparison showed more highly differentiated SNPs for freshwater-saltwater pairs than for geographically distant populations of the same

salinity (Kozak et al. 2014). Furthermore, a GO enrichment analysis was performed on outlier loci with the use of Infinium bead genotype data aligned to the transcriptome reference. The SNPs corresponded to enrichment of ion transport and cell junction genes, which are responsible for producing proteins such as claudin 4, claudin 10, claudin 17, and tight junction protein ZO-3, which are important controls for changes in gill permeability or gill remodeling in response to changes in salinity (Tipsmark et al. 2008, Kozak et al. 2014).

This study has two aims. First, I wanted to measure the extent of local adaptation to freshwater and saltwater habitats. Second, I wanted to measure the extent to which the salinity of the spawning environment of the parent affects the subsequent survival of offspring in different salinity conditions. To meet these goals, I collected fish from a freshwater and a saltwater population pair in North Florida, performed crosses in high salinity and low salinity conditions, and raised the offspring under various salinity conditions. This allowed me to determine the effects of population salinity, spawning salinity, and rearing salinity on offspring survival.

METHODS AND MATERIALS

Collection

Lucania parva were collected from one freshwater and one saltwater population in the Wakulla/North Florida River drainage system. Fish were collected using dipnets and seines from the two populations in Florida in June 2019. Freshwater *L. parva* were collected from Lower Bridge (LB) on the Wakulla River (0.2 ppt) and saltwater *L. parva* were collected at Lighthouse Pond (LHP) in the St. Mark's National Wildlife Refuge (23.3 ppt). Upon collection, fish were held in buckets containing water from the collection site and transported back to the University of Illinois at Urbana-Champaign.

Crosses

In the lab, fish were maintained in stock tanks in a temperature-controlled greenhouse. Adults were fed once per day with frozen *Artemia*. The Lower Bridge population was initially maintained at 2 ppt and was later transitioned to 15 ppt, and the Lighthouse Pond population was maintained at 30 ppt. For the water in the stock tanks and crosses, we used reverse osmosis (RO) water from a filtration system (AquaFx Barracuda 4 Stage RO/DI System, Winter Park, FL) to which RO Right (Kent Marine, Franklin, WI) and Instant Ocean Sea Salt (Spectrum Brands, Atlanta, GA) were added to achieve the desired conductivity and salinity. Salinity was measured with a YSI-63 salinity/conductivity meter (YSI Inc., Yellow Springs, OH).

For each population, I created 20 breeding pairs, of which half spawned in low salinity (2 ppt) and half spawned in high salinity (30 ppt) for a total of 40 crosses (10 LHP in low salinity, 10 LHP in high salinity, 10 LB in low salinity, and 10 LB in high salinity). Although 20 Lighthouse Pond breeding crosses were set up, some females died before producing enough eggs for all of the offspring water treatments.

Each pair was placed into a 109 liter tank containing 4-6 yarn mops as a spawning substrate and a sponge filter. Two or three mops were attached to PVC pipes to let them sink to the bottom of the tank, and the other mops were attached to small Styrofoam balls to let them float. Mops were checked every 2-3 days for eggs from 12 June 2019 to 18 September 2019. All the eggs from each tank were placed into a small plastic container with water from the tank and dilute methylene blue dye before being sorted into treatments.

Egg and larval survival

Eggs were checked under a dissecting scope for fertilization and developmental stage. Only living, fertilized eggs were placed into small tubs containing one of four water treatments: soft fresh water (30-50 microsiemens conductivity), hard fresh water (700-900 microsiemens conductivity), marine water (30 ppt), and hypermarine water (60 ppt). I chose hard freshwater and marine conditions because these represent the ends of the continuum of salinity in which *L. parva* is most often found. I also used a soft fresh water and a hypermarine treatment to create particularly challenging osmotic environments that would not be frequently experienced in nature (IAL and IUBS 1958, Fuller and Noa 2008). To create these water treatments, I used RO water. The soft fresh water was RO water. The hard fresh water was RO water with R/O Right. For the saltwater treatments, I added Instant Ocean to hard fresh water to the desired salinity. Dilute methylene blue dye was added to each of the water treatments to prevent fungal infections. Water treatments were verified using a YSI salinity/conductivity meter. I varied which water treatments clutches were assorted into to control for order effects. When placing clutches into treatments, usually no more than 10 eggs were placed into a treatment tub. Extra eggs were placed in another treatment. All eggs were maintained in a temperature-controlled room in the laboratory. Eggs and larvae were censused for survival, hatching, and eating about every 3 days for one month. Dead eggs and larvae were removed during the census. Larvae were fed newly hatched *Artemia*.

Data Analysis

The goal was to determine whether population of origin, spawning salinity, or offspring salinity affected offspring survival and whether these patterns were consistent with the

hypothesis of local adaptation or adaptive maternal effects (Figure 3.1). I used the proportion of offspring surviving to day 5, hatching, and eating as measures of offspring fitness. For each spawning pair, I calculated the total number of offspring in each treatment, the number of eggs surviving to day 5 post-fertilization, the number of larvae that hatched in each treatment, and the number of larvae surviving to successfully eat. If there were less than 5 eggs from any breeding pair in a treatment, then that replicate was excluded from the analysis to minimize sampling error. I also calculated the overall survival as the proportion of the total number of eggs to reach the eating stage. I also calculated the proportion of offspring that survived to each stage relative to the previous stage: the proportion of eggs that survived to day 5, the proportion of surviving eggs that hatched, and the proportion of hatched larvae that successfully ate.

I used generalized linear models to determine the effects of populations from different habitat salinities, spawning salinity, offspring salinity, and their interactions. The hypothesis of local adaptation predicts an interaction between habitat salinities and offspring salinity where survival is higher in fresh water for offspring whose parents came from a freshwater habitat and (vice versa) where offspring survival is higher in salt water for offspring whose parents came from a saltwater habitat. The hypothesis of maternal effects predicts an interaction between spawning salinity and offspring salinity where offspring spawned in fresh water have higher survival in fresh water than offspring spawned in salt water and (vice versa) where offspring spawned in salt water have higher survival in salt water than offspring spawned in fresh water. I used models that assumed a binomial distribution, where the proportion of offspring surviving was the dependent variable. I checked the models for overdispersion and corrected them by using the quasibinomial distribution throughout. I used type 3 analyses to examine the statistical significance of each factor in the car package in R. I used the ‘glm’ function throughout, and

specified the contrasts with the 'options = c("contr.sum", "contr.poly"))' statement. This is mandatory when analyzing unbalanced models with interactions.

RESULTS

Table 3.1 shows the number of eggs in each treatment used in this study. Lower Bridge population pairs laid more eggs overall compared to Lighthouse Pond pairs, as some of the Lighthouse Pond fish died before producing enough eggs for each treatment to be included in the analyses.

I found strong evidence that offspring salinity had large effects on multiple stages of survival (Table 3.2). Figures 3.2 and 3.3 show overall survival as a function of offspring salinity, spawning salinity, and their interaction. Surprisingly, overall survival (from egg to eating) was lowest in the hard fresh water, which was intended to simulate the conditions of a spring-fed river. Overall survival was highest in the marine habitat (86%) followed by the soft freshwater treatment (76%), hypermarine (66%), and hard freshwater treatments (52%). In addition to the overall effects of offspring salinity, I found that there was an effect of spawning salinity (i.e., the salinity in which the offspring were spawned) and an interaction between offspring salinity and spawning salinity. Overall survival was higher when offspring were spawned at 30 ppt (78%) in comparison to 2 ppt (63%).

The hypothesis of adaptive maternal effects predicts a statistically significant interaction between spawning salinity and offspring salinity, where offspring spawned in fresh water have higher fitness than offspring spawned in salt water and (vice versa) offspring spawned in salt water have higher fitness than offspring spawned in fresh water. While there was a statistically significant interaction between offspring salinity and spawning salinity (Table 3.2A), the

interaction likely occurred due to the fact that offspring had higher survival when spawned in salt water, except for offspring that were reared in marine (i.e., 30 ppt) conditions (Figure 3.3A). The hypothesis of local adaptation predicts a statistically significant interaction between population and offspring salinity, where offspring from the freshwater population have higher fitness in fresh water than offspring from the saltwater population and (vice versa) offspring from the saltwater population have higher fitness in salt water than offspring from the freshwater population. There was no statistically significant interaction between population and offspring salinity (Table 3.2A, $X^2 = 3.65$, $p = 0.302$), although the pattern of differential survival was in a direction that was consistent with local adaptation.

Analyses of survival across each stage show that offspring salinity had statistically significant effects on each stage except from fertilization to day 5 where spawning salinity had its largest effect (Tables 3.2B-D). There was also an interaction between population and spawning salinity on survival from day 5 to hatching where offspring from saltwater parents had higher survival than offspring from freshwater parents when spawned in salt water and vice versa (Figure 3.4). While this is consistent with local adaptation at a very early life stage, the interaction did not affect overall survival.

DISCUSSION

The goals of this study were (a) to test for the presence of local adaptation as a function of salinity in early embryo and larval survival and (b) to test for adaptive maternal effects as a function of salinity in early embryo and larval survival. In order for there to be local adaptation, average fitness must be higher for individuals from the local environment relative to individuals from a foreign environment (Schluter 2000). In this study, I tested for local adaptation as a

function of salinity in a pair of *L. parva* populations, hypothesizing that offspring from freshwater parents would outperform offspring from saltwater parents when raised in fresh water and vice-versa. However, I found little evidence for local adaptation. Instead, I found that the salinity in which offspring were reared (offspring salinity) had a large effect on offspring survival. There were also interactions between spawning salinity and offspring salinity and between population and spawning salinity. I discuss the implications of these results below.

Overall Effects of Water Chemistry

Contrary to our expectations, I found that offspring regardless of population or spawning salinity had reduced survival in hard fresh water and in hypermarine conditions. Surprisingly, I found high survival of *L. parva* offspring in the soft freshwater treatments. *L. parva* is a euryhaline species that can invade hard, fresh water (Lee et al. 1983, Fuller and Noa 2008). Dunson and Travis (1991) showed that adult *L. parva* can readily tolerate hard water (i.e., water with high ion concentrations), but that soft water with pH less than seven is deadly. Our soft water treatments were low in dissolved ions with a conductivity of 30-50 microsiemens and a pH of 7.4-7.6, possibly making the treatment less stressful than intended. Additionally, the high survival of embryos in soft water in the presence of methylene blue is not uncommon. Kozak et al. (2012) showed that embryos can have high survival in RO water in the presence of methylene blue, which traditionally has been used to prevent fungus infection of fish eggs (Khoo 2000). This high survival may be due to changes in water chemistry. The reduction in survival in the hypermarine (60 ppt) treatment may also be an indication of increased osmotic stress during early life stages. Still, 66% of offspring survived to the eating stage. *L. parva* adults have been shown to tolerate salinities of up to 80 ppt, but important osmoregulatory structures like gills are

still developing in eggs and during early larval stages (Guggino 1980, Alderdice 1988). Still, the overarching picture here is one of incredible tolerance by eggs and embryos of a wide range in salinities and water chemistries.

The Lack of Local Adaptation

The striking lack of adaptation is at odds with the population genomic analysis of Kozak et al. (2014) that found elevated levels of F_{ST} between freshwater and saltwater population pairs. Furthermore, there was evidence that genes important to osmoregulation were enriched. In chapter 2, I found evidence for a weak, but significant effect of salinity in a mantel analysis and principal component analysis. Hence, genomic data suggest differential selection as a function of salinity, while the salinity assays described here show no real signature of local adaptation. There are three possible reasons for this discrepancy.

The first reason for this discrepancy may simply be that the two populations I used are the two most closely related of all of the freshwater/saltwater population pairs analyzed in chapter 2. The F_{ST} for the Florida Gulf Coast freshwater-saltwater population pair used in this experiment was 0.029, compared to the Atlantic Coast population pair (0.186), and Suwannee River population pair (0.039). This suggests that the two populations share substantial gene flow, which may decrease levels of differentiation. While I did not find evidence for local adaption as a function of salinity in their population pair, it may be because of sampling. Testing more population pairs could provide more insight on the capability for local adaption in this species.

A second potential reason for this discrepancy may be that salinity poses its greatest challenges at other parts of the life cycle. Survival over the winter would include being exposed to colder temperatures, which can be challenging for euryhaline fish. Cold temperatures often

disrupt ion exchange mechanisms and can cause enzymes that are important for osmoregulation to perform poorly (Hochachka 1988, Kidder et al. 2006). In an experiment testing population variation in sailfin mollies as effects of salinity, temperature, and food, Trexler et al. (1990) found that female sailfin mollies (*Poecilia latipinna*) grew more slowly and matured later in colder temperatures at low salinities. Sailfin mollies maintained in field cages experienced lower overwinter survival in fresh water compared to salt water (Trexler et al. 1992). Similarly, overwinter survival to adulthood was lowest for *L. parva* in low salinities (Fuller et al. 2007). These studies suggest an interaction between salinity and temperature, where lower temperatures in low salinities are particularly challenging.

A third potential reason for this discrepancy may be that these habitats differ in other important properties that create parallel selection, but on traits other than salinity tolerance. Some other abiotic factors that affect *L. parva* distribution are temperature, oxygen, pH, and pollutants (Dunson and Travis 1991, Dunson et al. 1993). Salinity-temperature interactions in particular may help to explain why *L. parva* would invade springs and spring-fed rivers despite the challenge of overwintering in low salinities (Fuller and Noa 2008). The temperature of freshwater springs tends to have a surprisingly consistent temperature (21ºC) year round (McKinsey and Chapman 1998, Fuller et al. 2007). Additionally, biotic factors such as aquatic vegetation, predation, and food resources may covary with salinity across *L. parva* sites (Dunson and Travis 1991, Jordan 2002, Fuller and Noa 2008). Vegetation, predators, and food availability may create similar freshwater or saltwater habitats and communities across replicate drainages (Fuller and Noa 2008). Finally, heterospecifics that compete for resources with *L. parva* may favor either freshwater or saltwater environments (Dunson and Travis 1991, Fuller and Noa 2008). The bluefin killifish, *Lucania goodei,* is of particular note because it is closely related to

L. parva and is sympatric with *L. parva* in 12-19% of *L. goodei* sites in fresh and brackish water across Florida (Fuller and Noa 2008). Salinity can affect the competition coefficient between *L. parva*, *L. goodei,* and other species (Rowe and Dunson 1995). In competition experiments between *L. parva* and *L. goodei* in fresh water and brackish water, *L. parva* had greater weight gain in brackish water whereas *L. goodei* had greater weight gain in fresh water when in competition with the heterospecific species (Dunson and Travis 1991). The weight gain in *L. goodei* in brackish water was greater when competing with conspecific *L. goodei* than when competing with heterospecific *L. parva* (Dunson and Travis 1991). Thus, there may be parallel evolution among *L. parva* populations that are sympatric with *L. goodei* due to competition.

Maternal Effects

The limited osmoregulatory capabilities of fish in early life stages may also relate to the importance of maternal effects. Since euryhaline fish are most suited for environment with variable salinity, provisioning offspring depending on the spawning salinity may be an adaptive strategy to increase the survival of offspring until they are able to develop complex osmoregulatory structures (Alderdice 1988, Varsamos et al. 2005, Green 2008). I found some evidence for maternal effects (via significance of spawning salinity at day 5) in the early life stages of the offspring, though these effects may not be adaptive because offspring that were spawned in salt water tended to have higher survival regardless of offspring salinity treatments.

In the literature, maternal effects are receiving an increasing amount of attention across a wide variety of taxa, parental strategies, and environment types (Mousseau and Fox 1998, Green 2008, Burt et al. 2011, Murphy et al. 2014, Kruuk et al. 2015, Feiner et al. 2016). However, maternal effects as a function of salinity have been largely overlooked despite their potential

importance (Green 2008). Notably, Shikano and Fujio (1998) found that when gravid guppy mothers acclimated to sea water, salinity tolerance increased in the offspring. In mangrove rivulus (*Rivulus marmoratus*), fish reared in low salinity produced fewer, larger eggs that had higher hatching success and decreased time to hatching compared to fish reared in higher salinity (Lin and Dunson 1995).

Conclusions

While I found strong evidence of effects of water chemistry, and some evidence of maternal effects on offspring survival, there was little evidence for local adaptation as a function of salinity and little evidence that maternal effects were adaptive. These results highlight the incredible salinity tolerance of *L. parva* even during early life stages. They also suggest that the mechanism via which genetic differentiation occurs between freshwater-saltwater population pairs (chapter 2) is currently unknown. I suggest that overwinter survival is an understudied life-history stage that may be important to local adaptation.

TABLES AND FIGURES

Table 3.1. Number of eggs in each treatment that were included in the analysis.

Population	Spawning Salinity	Offspring Salinity	Number of Breeding Pairs	Number of Eggs
Lower Bridge	Fresh	Soft fresh	10	275
		Hard fresh	10	282
		30 ppt	10	318
		60 ppt	10	325
	Salt	Soft fresh	10	192
		Hard fresh	10	202
		30 ppt	10	165
		60 ppt	10	170
Lighthouse Pond	Fresh	Soft fresh	8	125
		Hard fresh	8	144
		30 ppt	8	137
		60 ppt	8	113
	Salt	Soft fresh	8	155
		Hard fresh	8	158
		30 ppt	8	172
		60 ppt	6	139

Table 3.2A. Overall survival to eating stage (number of larvae eating/number of eggs).

	df	X^2	p
Population	1	0.137	0.712
Offspring Salinity	3	58.292	**<0.001**
Spawning Salinity	1	14.267	**<0.001**
Population:Offspring Salinity	3	3.651	0.302
Population:Spawning Salinity	1	2.619	0.106
Offspring Salinity:Spawning Salinity	3	10.374	**0.016**
Population:Offspring Salinity:Spawning Salinity	3	0.644	0.886

Table 3.2B. Survival to day 5 post-fertilization (number of eggs alive day 5/number of eggs fertilized).

	DF	X^2	p
Population	1	0.0004	0.985
Offspring Salinity	3	6.350	0.096
Spawning Salinity	1	10.500	**0.001**
Population:Offspring Salinity	3	4.980	0.173
Population:Spawning Salinity	1	0.043	0.835
Offspring Salinity:Spawning Salinity	3	3.200	0.362
Population:Offspring Salinity:Spawning Salinity	3	1.070	0.785

Table 3.2C. Survival from day 5 to hatching (number of larvae hatched/ number of eggs alive day 5).

	DF	X^2	p
Population	1	2.297	0.114
Offspring Salinity	3	27.900	**<0.001**
Spawning Salinity	1	2.816	0.093
Population:Offspring Salinity	3	5.824	0.121
Population:Spawning Salinity	1	7.948	**0.004**
Offspring Salinity:Spawning Salinity	3	7.559	0.056
Population:Offspring Salinity:Spawning Salinity	3	4.300	0.231

Table 3.2D. Survival from hatching to eating (number of larvae eating/number hatched).

	DF	X^2	p
Population	1	0.397	0.529
Offspring Salinity	3	69.359	**<0.001**
Spawning Salinity	1	3.072	0.080
Population:Offspring Salinity	3	2.758	0.430
Population:Spawning Salinity	1	0.051	0.820
Offspring Salinity:Spawning Salinity	3	6.310	0.097
Population:Offspring Salinity:Spawning Salinity	3	1.845	0.605

Figure 3.1. Experimental design illustrating population salinities, spawning salinities, and offspring salinity treatments.

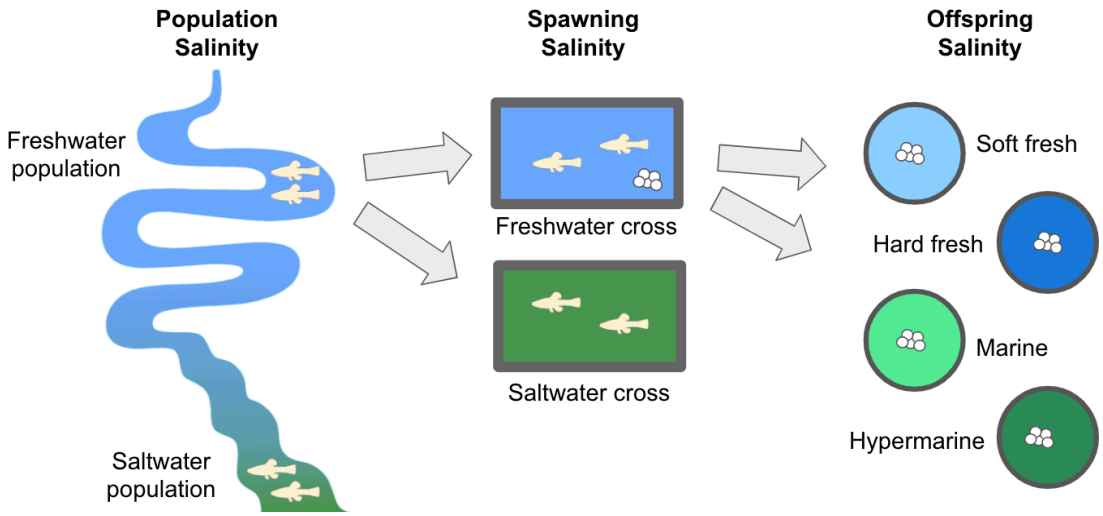


Figure 3.2. Overall survivorship curves of *L. parva* offspring due to the interaction between offspring salinity and (A) spawning salinity and (B) population.

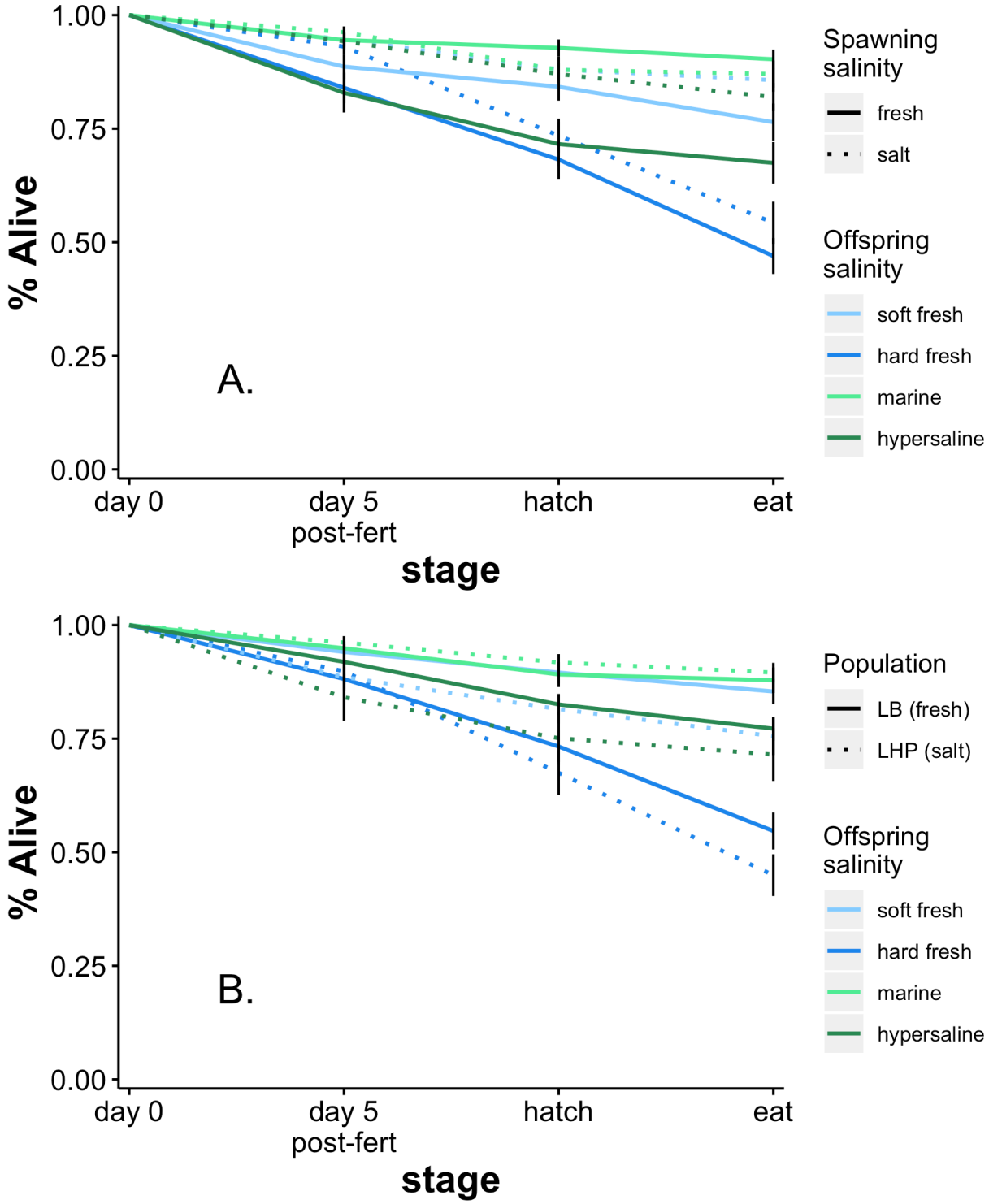


Figure 3.3. Overall survival of offspring to from eggs to eating by offspring salinity and (A) spawning salinity and (B) population. Means ± SE are shown.

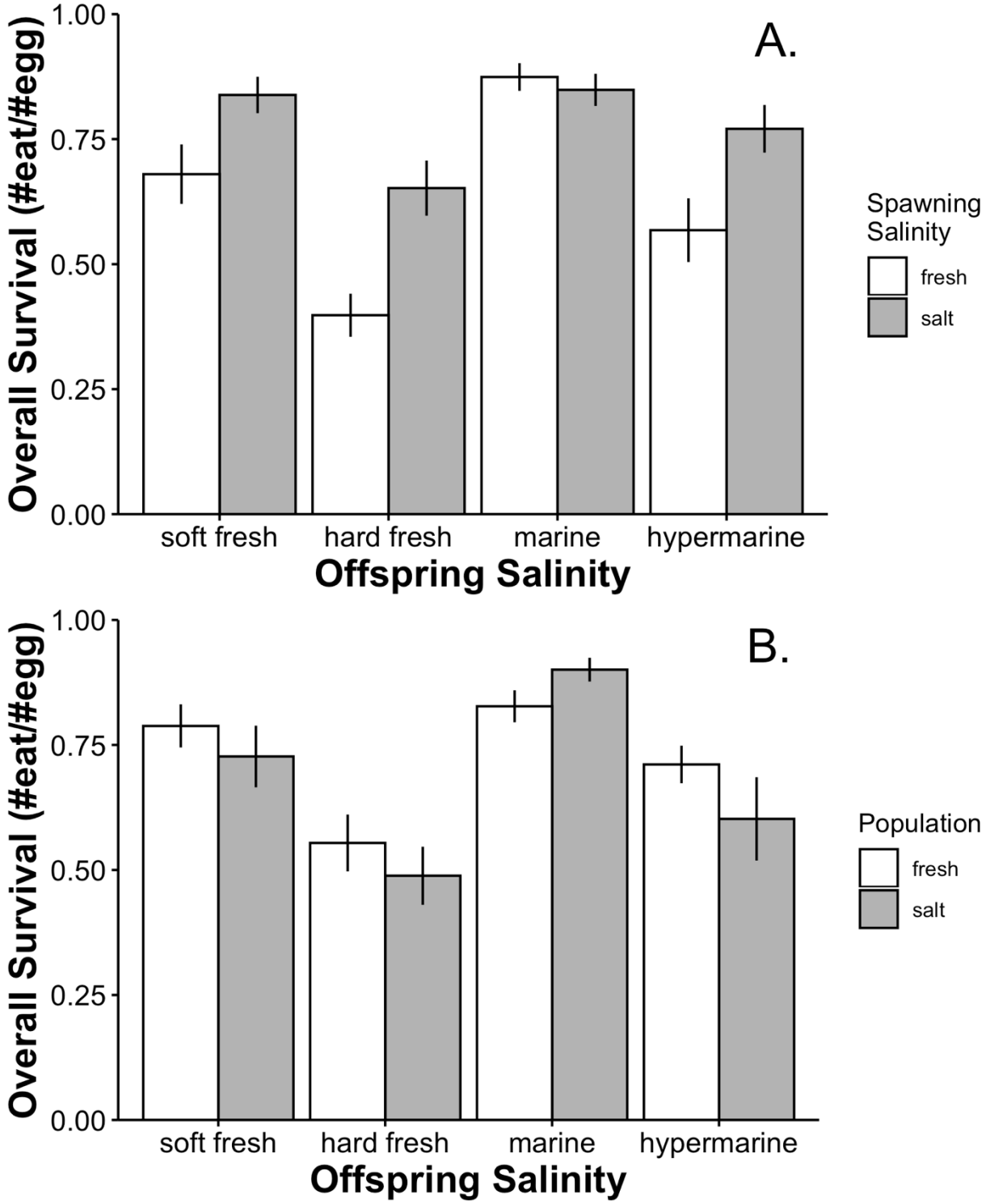
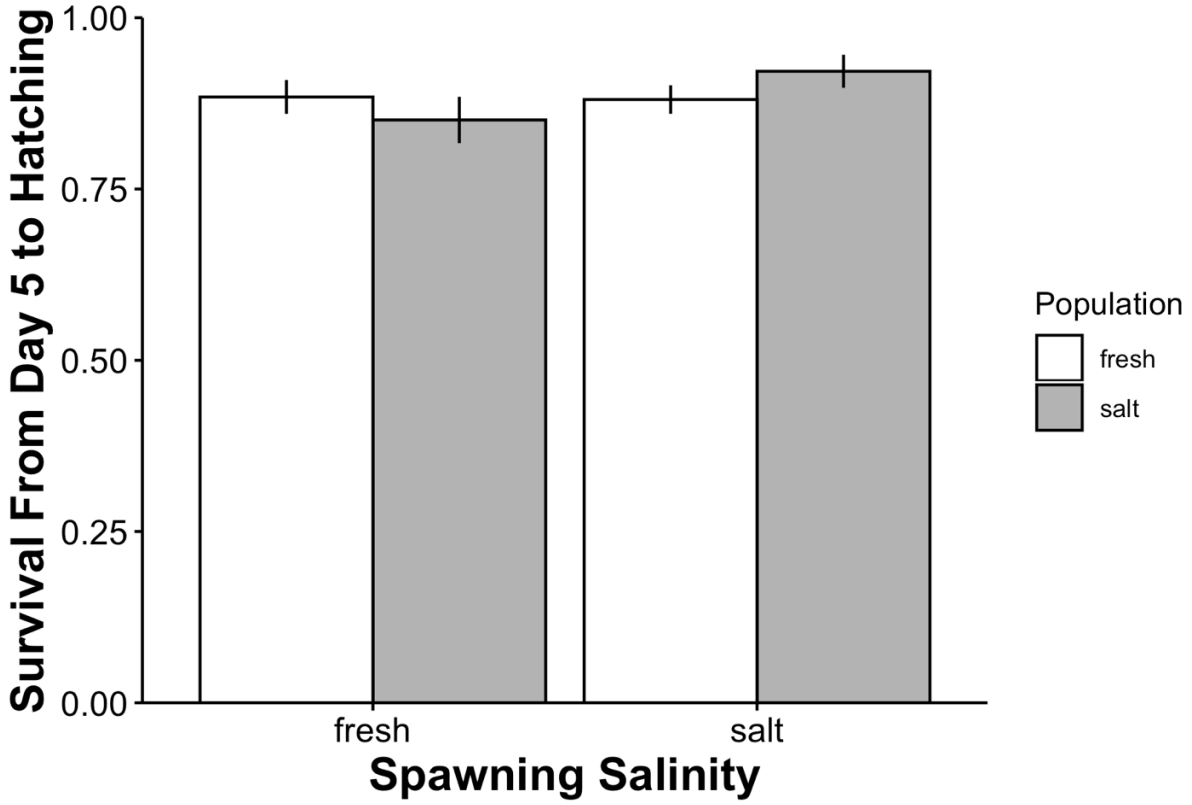


Figure 3.4. Survival from day 5 to hatching as a function of the interaction between spawning salinity and population. Means ± SE are shown.



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