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The Role of Developmental Plasticity in Response to Salinity on the Evolution of Alternative Reproductive Tactics in the Sailfin Molly, *Poecilia Latipinna* (Poeciliidae: Poecilia: Mollienesia)

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THE ROLE OF DEVELOPMENTAL PLASTICITY IN RESPONSE TO SALINITY ON
THE EVOLUTION OF ALTERNATIVE REPRODUCTIVE TACTICS IN THE SAILFIN
MOLLY, *POECILIA LATIPINNA* (POECILIIDAE: *POECILIA: MOLLIENESIA*)

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

by
Kelly Elise Hogan
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Accepted by:
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ABSTRACT

Variability in life history and morphology across conspecific populations is a widespread phenomenon and may be attributed to life history trade-offs in response to environmental variation. Such trade-offs between growth, maintenance, and reproduction often occur via developmental plasticity, which allows organisms to shift their developmental trajectories to maximize fitness in a given environment. Sailfin mollies (*Poecilia latipinna*) exhibit interpopulation variation in life history and morphological traits, and inhabit springs, tidal creeks, and estuaries that range from freshwater to seawater. Male size at maturity is smaller on average in freshwater springs than in brackish water marshes where these fish are most abundant. Such variation influences male fitness, because male size is fixed at maturity, highly variable, and correlated with the expression of alternative reproductive tactics. Large males erect an exaggerated dorsal sailfin in courtship displays, whereas small males perform sneaking mating behavior. In order to determine if developmental plasticity plays a role in interpopulation variation and the maintenance of size-associated mating tactics, I used a split brood design to rear siblings in low salinity (2 ppt) and brackish water (20 ppt) until sexual maturity. I found that growth and maturation rates of males and females did not differ in response to salinity. Mortality rate was somewhat higher in 20 ppt relative to 2 ppt. Females were smaller at maturity in brackish water, though their body condition did not differ in response to salinity treatment. Males also responded plastically to salinity environment, though the direction and magnitude depended largely on family genotype. Males exhibited better body condition in 20 than 2 ppt. The relative size (to body area) of the sexual ornament (dorsal sailfin) was smaller for males reared in low salinity than

brackish water, suggesting that males in brackish water could allocate more energy resources towards producing larger sexual ornaments. Because males produce smaller sexual ornaments and have reduced body condition in low salinity, small male body size and sneaking mating tactics may be favored in freshwater populations. Such a shift in the balance between natural and sexual selection between salinity environments may help to explain interpopulation variation in *P. latipinna*.

DEDICATION

I dedicate this thesis to my parents, Randy and Sara Hogan. Their unwavering support and encouragement has afforded me the opportunity to pursue my passion for biology. Through my dad's example, I have learned that a rewarding career stems from great passion, perseverance, and confidence. Through my mom's example, I have learned that curiosity and humility are qualities of the utmost importance. Both have taught me that maintaining happiness and humor is most important of all. I hope to emulate all of these characteristics in my career and in life.

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CHAPTER ONE

UNDERSTANDING THE ROLE OF PHENOTYPIC PLASTICITY IN THE PRODUCTION OF ALTERNATIVE REPRODUCTIVE TACTICS

INTRODUCTION

Polymorphic traits that repeatedly occur within a life stage or population are widespread across taxa and may be expressed in life-history traits, physiology, morphology, and behavior (Moran 1992; West-Eberhard 1986, 2003). These alternative phenotypes are often associated with specializations in reproduction, feeding, dispersal, or predator avoidance (West-Eberhard 2003; Leimar 2009). Understanding the origin and maintenance of alternative phenotypes within a species provides clues to the evolutionary history of such taxa because these polymorphisms may represent evolutionary phases leading to speciation. Novel alternative traits within a population may evolve to fixation or be maintained indefinitely as polymorphisms. This could allow for the evolution of new adaptive specializations via the elaboration of the novel trait without the elimination of the established trait (West-Eberhard 2003). Alternative phenotypes associated with mating signals are of particular interest because selection on these traits should be strong and can lead to speciation via reproductive isolation (Panhuis *et al.* 2001).

Within a sex, alternative phenotypes are often associated with reproductive tactics (Andersson 1994; Gross 1996). These alternative reproductive tactics (ARTs) arise in populations where sexual selection is strong and relatively few individuals

secure mates (Shuster 2010). ARTs have been described in most animal taxa, including invertebrates (e.g., freshwater prawns (*Macrobrachium rosenbergii*): Ra'anan & Sagi 1985); beetles (*Onthophagus taurus*): Moczek 1998; marine isopods (*Paracerceis sculpta*): Shuster & Wade 2003; mites (*Rhizoglyphus echinopus*): Tomkins *et al.* 2011), reptiles (e.g., side-blotched lizards (*Uta stansburiana*): Sinervo & Lively 1996), birds (e.g., ruff (*Philomachus pugnax*): Lank *et al.* 1995), mammals (e.g., elephant seals (*Mirounga angustirostris*): Le Boeuf 1974; red deer (*Cervus elaphus*): Clutton-Brock *et al.* 1979), and fishes (e.g., cichlids (*Lamprologus callipterus*): Schütz *et al.* 2010; salmon (*Salmo salar*): Garant *et al.* 2003; bluegill sunfish (*Lepomis macrochirus*): Gross 1991, Neff *et al.* 2003; guppies (*Poecilia reticulata*): Godin 1995; swordtails (*Xiphophorus nigrensis*): Zimmerer & Kallman 1989). A notable example of ARTs occur in beetles (*Onthophagus taurus*) where male phenotype is correlated with mating strategy; long-horned males defend territories and hornless males sneak copulations (Moczek 1998). Body size and color polymorphism in male side-blotched lizards (*Uta stansburiana*) is related to mating behavior; large, orange-throated males defend many females on large territories, intermediate-sized, blue-throated males mate-guard a single female, and small, yellow-throated males patrol large home ranges and sneak copulations (Sinervo & Lively 1996; Sinervo *et al.* 2000). Similarly, marine isopod (*Paracerceis sculpta*) males fall into three distinct color and body size morphs that correspond to bourgeois (i.e., territorial) and parasitic (i.e., sneaking) mating tactics (Shuster 1987). These examples illustrate the ubiquity of ARTs across taxa.

Although ARTs are common in animals, how they are maintained in many taxa is still unknown (Rio-Cardenas & Morris 2011). There are two leading, mutually exclusive hypotheses that have been suggested to explain the origin and maintenance of ARTs:

game theory and “best of a bad job.” Game theory argues that alternative tactics are stabilized because they yield similar Darwinian fitnesses (Taborsky 1998), and therefore represent an evolutionary stable strategy (ESS). When an ESS is established in a population, no other strategy can invade, and as such, ARTs are maintained by negative frequency-dependent selection (Maynard Smith 1982; Andersson 1994). This theory assumes that alternative tactics are either purely genetic or may be correctly adopted by individuals that are phenotypically flexible (Andersson 1994). Sinervo & Lively (1996) provide empirical support for this model: frequencies of three alternative male morphs of side-blotched lizards oscillated concordantly with expectations of negative frequency-dependent models. Further, ARTs in these lizards are genetically determined (Sinervo *et al.* 2001; 2006), and therefore, align with the assumptions of the game theory framework.

However, ARTs are not always genetically fixed. Mating tactics may vary with individual condition or size (Dawkins 1980; Andersson 1994; West-Eberhard 2003). For example, young satellite males opportunistically mate with females in a competitor’s harem until they are large enough to establish their own territories (e.g., northern elephant seals (*Mirounga angustirostris*): Le Boeuf 1974; red deer (*Cervus elaphus*): Clutton-Brock *et al.* 1979). ARTs are also common in males that cease to grow after maturation, yet differ significantly in size and morphology at maturity. For example, larger male yellow dung flies (*Scathophaga stercoraria*) fight for access to female-preferred cow dung patties, whereas smaller males must attempt to find mates in the surrounding grass areas (Pitnick *et al.* 2009). Similarly, horned beetles (*Onthophagus taurus*) differ in horn ornamentation, and long-horned males defend territories while hornless males sneak copulations (Moczek 1998). Horn size is determined by nutrition

during ontogeny, suggesting that these exaggerated sexually-selected traits are reliable signals of male quality (Emlen *et al.* 2012). Instead of representing ARTs with equal fitnesses, these examples describe scenarios in which low-quality males may be making “the best of a bad job” (Dawkins 1980). Individuals with inferior competitive abilities maximize fitness by adopting an alternative mating strategy because some mating opportunities are better than none (Dawkins 1980; Andersson 1994 Shuster 2010).

From a proximate level, ARTs may arise as a result of underlying genetic control (G), environmental conditions (E), or plasticity (GxE) (e.g., guppies (*P. reticulata*): Hughes *et al.* 2005; sailfin mollies (*P. latipinna*): Travis & Woodward 1989; Fraser *et al.* 2014). Virtually all quantitative traits are influenced by both genetic and environmental factors (Scheiner 1993), yet understanding the evolutionary significance of phenotypic plasticity (GxE) in producing heritable phenotypic variation is a challenging aspect of the study of ARTs. Identifying the role of plasticity in the origin and maintenance of ARTs is important because its presence can complicate estimations of genetic variation and alter the outcome of selection in natural populations (Trexler 1989). Indeed, Leimar (2009) argues for a unified framework of genetic and environmental cues in determining phenotypes during development, because genes and the environment play similar roles as determinants of development.

Developmental plasticity may play an integral role in the evolution and maintenance of ARTs, occurring when an organism modifies its developmental trajectory in response to environmental conditions (Moczek *et al.* 2011). Specifically, environmental conditions during ontogeny can act as cues that initiate alternative developmental pathways. This mechanism is known as a “developmental switch” and leads to shifts in life history traits, morphology, physiology, and behavior (West-Eberhard

1989; Pfennig 1990; Nijhout 2003). Developmental switches may be an adaptive response to environmental stressors or conversely, an unavoidable consequence of environmental heterogeneity (Badyaev 2005; Ghalambor *et al.* 2007; Pfennig *et al.* 2010). Environmental stressors during ontogeny can lead to alternative phenotypes, such as early maturation in response to habitat desiccation (e.g., Newman 1992; Denver 1997; Denver *et al.* 1998; Johansson & Richter-Boix 2013) and predator-induced defense mechanisms (e.g., Spitze 1992; Boersma *et al.* 1998; Benard 2004). Fewer studies have investigated the role of developmental plasticity in producing ARTs.

From a theoretical standpoint, the ART literature has described a mechanism similar to the “developmental switch,” in which ART expression may be determined by heritable threshold responses to environmental variation during development (Taborsky & Brockmann 2010). This threshold or “switch point” mechanism has been described in the well-studied horned beetle system. Male beetles that do not reach a threshold body size will develop reduced horns relative to their large-bodied territorial competitors and gain access to females by digging side tunnels in order to bypass guarding males (Emlen 1997; Moczek & Emlen 2000). Further, Emlen (1996) demonstrated that this size-dependent life history switch point was heritable using artificial selection experiments. Specifically, small- and large-horned males were mated to random females resulting in shifts in size-dependent switch points for each sire size treatment (Emlen 1996). However, nutritional quality during development alters the allometry of horn size, whereby well-fed male offspring develop larger horns relative to body size than do poorly fed offspring (Emlen *et al.* 2012), illustrating the importance of genes and the developmental environment in producing size-dependent ARTs. Identifying the role of

developmental plasticity in producing ARTs in other model systems will be necessary to better understand this intriguing mating system.

Taborsky (1998) identified three major areas of ART research: determining (1) the relative degree to which genetic or environmental variation determines phenotype, (2) the relative degree to which plasticity influences phenotypes, and (3) the selective regimes that maintain or erode persistence of these alternative phenotypes. My thesis research addressed the first two major areas of ART research using the sailfin molly fish (*P. latipinna*). Specifically, my research focused on quantifying the relative importance of genes (G), environment (E), and phenotypic plasticity (GxE) in producing ARTs. Only after we have identified the proximate causation of ARTs can we begin to answer the ultimate question of how ARTs are maintained evolutionarily.

THE STUDY SYSTEM

The livebearing fish family, Poeciliidae (e.g., guppies, swordtails, and mollies), offers an ideal study system in which to examine the interplay between genes, the environment, and their interaction (i.e., plasticity) in producing ARTs. Poeciliids are known for their extensive diversity in male sexual traits such as body size, fin ornamentation, coloration, and mating behaviors, with males often having exaggerated secondary sexual ornaments such as coloration in guppies, caudal fin extensions in swordtails, and enlarged dorsal fins in sailfin mollies (Farr 1989). In swordtails and sailfin mollies, variation in body size and the size of these ornaments is often associated with the alternative mating strategies of courtship and sneaker behaviors (Farr *et al.* 1986; Zimmerer & Kallman 1989; Ptacek & Travis 1996). These male signaling traits are

influenced by both genetic and environmental factors. For example, body size (standard length (SL)) in males is heritable to some degree (e.g., swordtails: Kallman & Borkoski 1978; Borowsky 1987; Zimmerer & Kallman 1989; Lampert *et al.* 2010; mollies: Travis 1994), but is also influenced by environmental conditions during ontogeny such as the social environment (e.g., platyfish: Borowsky 1978; swordtails: Walling *et al.* 2007; guppies: Campton & Gall 1988; Magellan & Magurran 2009; mollies: Davidson & Hogan, unpubl. data).

My thesis research focused on the sailfin molly (Poeciliidae: *Poecilia*: *Mollienesia*: *Poecilia latipinna*), which is native to coastal regions along the Atlantic Ocean and Gulf of Mexico, ranging from South Carolina in the north into Veracruz, Mexico at the southernmost part of its geographic distribution (Nordlie *et al.* 1992; Ptacek & Breden 1998; Breden *et al.* 1999). Mollies are found naturally in habitats that range from freshwater (0 ppt) to seawater (32 ppt) estuaries (Rosen & Bailey 1963), and therefore, are considered to be euryhaline (e.g. Gunter 1950; Large 1985; reviewed in Meffe & Snelson 1989; Trexler *et al.* 1990;). Indeed, laboratory studies have shown that *P. latipinna* can tolerate a wide range of salinities from freshwater to hypersaline water (65-80 ppt) (Nordlie *et al.* 1992; Gonzalez *et al.* 2005), allowing them to occupy the largest geographic range of the four sailfin molly species: *P. latipinna*, *P. latipunctata*, *P. velifera*, and *P. petenensis* (Figure 1.2).

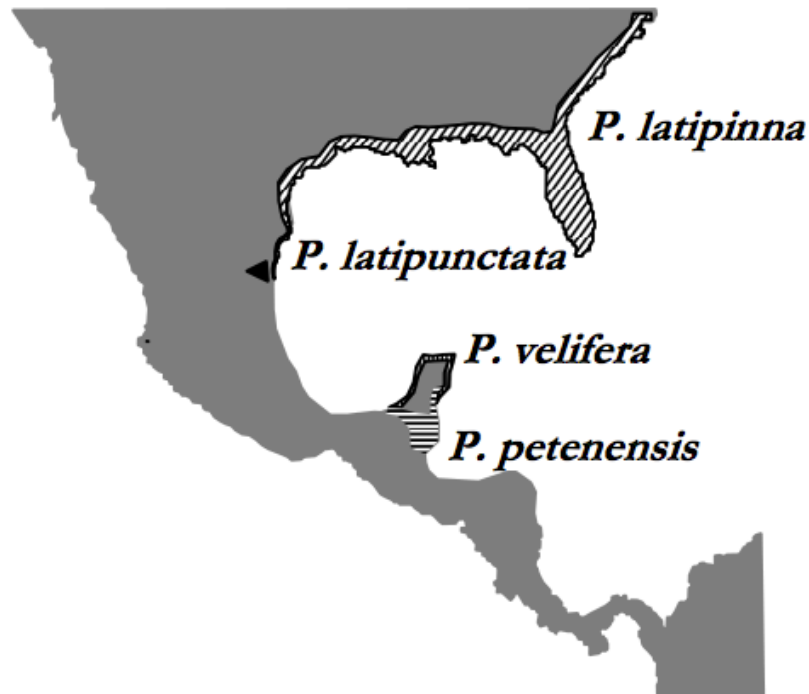


Figure 1.1. Map of North and Central America showing the distribution and range of four described species of sailfin mollies (Figure from Seda 2010).

Like all poeciliids, mollies are characterized by reproduction via internal fertilization (Farr *et al.* 1986). At the onset of sexual maturity in male poeciliids, the anal fin fuses into a copulatory organ termed the gonopodium, which is used to transfer sperm to the female's gonopore (Cummings 1943; Constantz 1989). Sailfin mollies are sexually dimorphic, and age and size at maturity in males (standard length (SL): measurement from the tip of the snout to the end of the caudal peduncle) is highly variable within and between populations (Snelson 1985; Ptacek & Travis 1996; Seda *et al.* 2012). Further, males grow minimally once mature, so small males never reach the size of larger males (Snelson 1982, 1984; Figure 1.2).



Figure 1.2. Small (25 mm; left) and large (50 mm; right) male *P. latipinna* from Steve's Ditch, Wakulla County, Florida.

Size variation in males has a genetic component, hypothesized to be inherited on the Y-chromosome (Travis 1994). The genetic architecture of male size at maturity has been studied extensively in the closely related genus *Xiphophorus* (swordtail fishes) where for several species (e.g., *X. nigrensis* and *X. multilineatus*), multiple alleles at the Y-linked pituitary (*P*) locus code for male size and mating behaviors by controlling the onset of sexual maturity (Kallman & Borkoski 1978; Borowsky 1987; Zimmerer & Kallman 1989). Specifically, early-maturing males are smaller and rely on sneak mating whereas late-maturing males are larger and perform courtship displays in order to gain female cooperation during copulation. As in *Xiphophorus*, male size of sailfin mollies is correlated with sexual behavior in which larger males erect an enlarged dorsal fin in a courtship display to elicit female cooperation during mating, and smaller males rely primarily on forced copulation attempts termed gonopodial thrusts (Farr & Travis 1986; Ptacek & Travis 1996; Seda *et al.* 2012). The size of the dorsal sailfin is strongly positively allometric with SL, and its expression is influenced by both genetic and environmental factors (Ptacek 2002; Hankison & Ptacek 2007). Indeed, there is

considerable additive genetic variance for dorsal fin size that maintains polymorphism within populations for this trait, at least in a related species of sailfin molly (*P. velifera*: Loveless *et al.* 2009). Finally, females prefer males with the largest lateral projection area (LPA: body area + dorsal and caudal fin areas), and can differentiate between very slight differences in male LPA (MacLaren *et al.* 2004; Ptacek & Travis 1997), making dorsal fin size a clear target of sexual selection.

Variation in life history and morphological traits of poeciliid fishes is also influenced by environmental factors. For example, temperature has been found to influence interbrood interval (Snelson *et al.* 1986) and biomass allocation (McManus & Travis 1998) in sailfin mollies, as well as age and size at maturity in mosquitofish (*Gambusia holbrooki*: Meffe 1992). Diet has been shown to influence growth rate and body size across poeciliid species (swordtails: Kruger *et al.* 2001; James & Sampath 2004; Ling *et al.* 2006; guppies: Dahlgren 1980; Reznick 1982, 1983, 1990; Reznick & Bryga 1987; Hughes *et al.* 2005; mollies: Tekelioglu *et al.* 2005). Further, preliminary data in our lab suggest that diet (i.e., protein level) during ontogeny influences the size of the male dorsal sailfin. Specifically, males reared on a high-protein diet have larger dorsal fins relative to their body size than males reared on a low-protein diet (Lange 2013). These findings suggest that the developmental environment plays an important role in final size and shape of male sailfin mollies.

Salinity may be an especially relevant environmental factor to consider when identifying the determinants of phenotypic variation in sailfin mollies, because mollies can be found naturally in habitats that range from freshwater (0 ppt) to seawater (32 ppt) estuaries (Rosen & Bailey 1963). Despite this tolerance, experimental evidence suggests that *P. latipinna* is best adapted to brackish water. Juveniles of *P. latipinna*

were found to grow faster (Zimmerer 1983) and females produced larger broods (Kumaraguru vasagam *et al.* 2005) when reared in brackish water relative to freshwater. Indeed, an examination of osmotic balance in *P. latipinna* has shown that mollies are best adapted physiologically to brackish water (Evans 1973, 1975; Gustafson 1981). In a reciprocal transplant field experiment, Trexler & Travis (1990) found that female and male *P. latipinna* grew more slowly and were older and smaller at maturity when reared in freshwater ponds relative to brackish water ponds regardless of population of origin. In a corresponding laboratory study, Trexler *et al.* (1990) found that both sexes matured later and at larger body sizes (SL) when reared in low salinity (2 ppt) relative to higher salinity (20 ppt). Nordlie *et al.* (1992) found that sailfin mollies from brackish water populations tolerated higher salinities better than those from freshwater populations. Araújo & Monteiro (2013) found that *P. vivipara* from a brackish water population grew faster but suffered higher mortality rates when reared in freshwater relative to a native freshwater population. Taken together, these studies suggest that sailfin molly populations along a salinity gradient can become locally adapted to fresh or brackish water, resulting in osmoregulatory trade-offs (Velotta *et al.* 2015).

At the physiological level, the gill is largely responsible for osmoregulation in euryhaline fishes (Evans *et al.* 2005). Specifically, suitable osmolality (electrolyte-water balance) in the blood and tissues is maintained through ionic transport mechanisms in the gill epithelium (Evans *et al.* 2005). Research in the gill structure of the closely related killifish (*Fundulus sp.*) suggests that rapid changes in ion transport pathways and morphology of the gill epithelium itself occur when these fishes are introduced into a stressful osmotic environment (reviewed in Evans *et al.* 2005). Similarly, Yang *et al.* (2009) found that activity in the branchial Na⁺-K⁺-ATPase (NKA) pathway, which is

responsible for ion secretion in the gill epithelium, increased in sailfin mollies that were introduced into full-strength seawater. Further, stress indicators were higher in mollies that were acclimated to seawater relative to those acclimated to freshwater or brackish water (Yang *et al.* 2009), suggesting that seawater is more stressful to sailfin mollies than brackish or freshwater. Gonzalez *et al.* (2005) found that oxygen consumption rate and metabolism in sailfin mollies increased as hyper-salinity increased. In summary, it appears that extreme salinities at both ends of spectrum: fresh and seawater, are not an ideal osmotic environment for sailfin mollies and may have impacts on growth and development.

GOALS AND OBJECTIVES OF MY STUDY

The goal of my thesis research was to quantify the degree to which genes (G), environment (E), and developmental plasticity (GxE) influence life history and morphological traits in response to salinity environment in the sailfin molly fish, *Poecilia latipinna*. In order to answer these questions, I conducted a full-sibling split brood complete block latin square design in which I reared full-siblings in low salinity (2 ppt) or brackish water (20 ppt) to investigate how salinity environment during ontogeny influences a suite of life history and morphological traits (mortality and growth rate, body condition, age, mass, length, and dorsal fin size at maturity). By rearing the offspring of mollies from a brackish water population (Steve's Ditch, Wakulla County, Florida) in low salinity (2 ppt) or brackish water (20 ppt), I was able to identify the relative contributions of (1) sire size (proxy for genetic effects), (2) salinity environment, and (3) their interaction (i.e., plasticity) on the expression of life history and morphological traits

associated with ARTs.

In total, I reared 309 fish (159 male, 150 females) from 12 families in which 123 had small sires (<30mm) and 186 had large sires (>48mm). This research resulted in measures of life history traits at the family and individual level. At the family level, growth rates were compared between treatments using a REML model to test the main effect of salinity treatment, as well as potential covariates: average temperature, average photoperiod, and cumulative population density. Family within sire size class and an interaction between family and sire size class were included as random effects.

Cumulative population density was measured as the sum of individuals in a tank across all census periods. Survival and maturation rates of males and females were assessed using the Kaplan-Meier method and compared using a log-rank chi-square test. At the individual level, size, approximate age (estimated to nearest 3 weeks), and wet mass at maturity were compared between treatments using a REML model to test the main effect of salinity treatment, sire size class, and their interaction, as well as two covariates: photoperiod and temperature. Family within sire size class and an interaction between family and sire size class were included as random effects. A linear regression between the natural log (ln) transformed standard length and natural log (ln) transformed mass was used as a measure of body condition, and was compared using a REML model to test for differences due to SL, salinity, and SL by salinity (a measurement of linear slope differences). Additionally, the allometry of two male morphological characters (i.e., dorsal fin area, caudal fin area) in response to salinity environment was assessed using linear regression between the natural log (ln) transformed trait and natural log (ln) transformed body area (BA). Adjusted r-square values, slope, and 95% confidence intervals of the slope were calculated and compared using a REML model to test for differences due to

BA, salinity, and BA by salinity (a measurement of linear slope differences).

The results of my thesis research will contribute to our understanding of how the developmental environment influences the origin of ARTs in natural populations. Specifically, if developmental environment is found to strongly influence life history variation, then natural populations that vary in abiotic factors should display similar differences in these traits. Therefore, interpopulation variation in phenotype due to gene-environment interactions may represent evolutionary phases leading to population divergence and ultimately, speciation. If I find that developmental environment does not influence life history variation, then ARTs may be strongly genetically linked in this species and may be maintained by negative frequency-dependent selection (Rios-Cardenas & Morris 2011). Aided by the results of my findings, future research should focus on identifying the selective regimes that maintain or erode ARTs in the sailfin molly study system.

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CHAPTER TWO

DEVELOPMENTAL PLASTICITY IN LIFE HISTORY AND MORPHOLOGY IN RESPONSE TO SALINITY ENVIRONMENT IN THE SAILFIN MOLLY (*POECILIA LATIPINNA*)

INTRODUCTION

Variability in life history and morphology across conspecific populations is a taxonomically widespread phenomenon (Trexler & Travis 1990), and may be largely attributed to life history trade-offs in response to environmental variation (Stearns 1992). Such trade-offs occur when an individual partitions finite energy resources between growth, maintenance, storage, and reproduction (Stearns 1992; Roff 2002). Maintaining homeostasis in stressful environments requires a substantial portion of this energy budget, leaving less for other functions such as growth and reproduction (Sibly & Calow 1989). Resource allocation for survival at the expense of other physiological functions alters the developmental trajectory of traits that are important determinants of reproductive success, including age and size at maturity (Stearns & Koella 1986; Stearns 1992; Zera & Harshman 2001) and condition-dependent sexual ornaments (Emlen *et al.* 2012). Therefore, populations that experience environmental stressors may exhibit life history and morphological divergence from conspecific populations in less stressful environments (e.g., Gomes & Monteiro 2008; Araújo & Monteiro 2013; Martin *et al.* 2009).

For fishes, salinity represents a major selective force that governs their physiology, life history, and habitat distribution (Boeuf & Payan 2001; Kültz 2015) by imposing trade-offs between maintenance and growth as a result of osmoregulation under different salinity conditions (Velotta *et al.* 2015). Estuarine habitats experience daily and seasonal fluctuations in salinity ranging from freshwater to even hypersaline conditions (Bamber & Henderson 1988). Consequently, euryhalinity (i.e., the ability to osmoregulate in both fresh and saltwater environments) in fishes is rare (Schultz & McCormick 2013). In those species that are euryhaline, higher salinity often increases growth hormone levels (Deane & Woo 2009), food intake, and conversion rates (Boeuf & Payan 2001). Despite these benefits, many euryhaline fishes have invaded freshwater habitats (Lee & Bell 1999; Schultz & McCormick 2013). Indeed, Bamber & Henderson (1988) suggest that selection for plasticity in euryhaline fish has pre-adapted them to living in freshwater habitats. In spite of their tolerance for low salinity, euryhaline species often exhibit reduced size and reproduction (e.g., *Poecilia vivipara*: Gomes & Monteiro 2007; Araújo & Monteiro 2013; *Poecilia latipinna*: Trexler & Travis 1990; Martin *et al.* 2009), or delayed maturation (*P. latipinna*: Trexler *et al.* 1990) in freshwater. Since osmoregulation requires as much as 50 percent of the total energy budget in fishes (Boeuf & Payan 2001), physiological trade-offs between maintenance and growth likely result in the phenotypic variation often observed in species inhabiting salinity gradients.

The sailfin molly (*P. latipinna*) offers an excellent fish system in which to explore how plasticity in response to salinity changes might influence expression of life history and morphology. This saltmarsh species is found naturally in habitats that range from freshwater to seawater in coastal regions along the Atlantic Ocean and Gulf of Mexico, ranging from South Carolina in the north into Veracruz, Mexico at the southernmost part

of its geographic distribution (Rosen & Bailey 1963; Ptacek & Breden 1998). Mollies tolerate a wide range of salinities from freshwater to hypersaline (Nordlie *et al.* 1992; Gonzalez *et al.* 2005). Despite this wide salinity tolerance, *P. latipinna* is best adapted physiologically to brackish water (Evans 1973, 1975), and is most abundant in brackish habitats of saltmarshes and estuaries with influx from freshwater tidal creeks (Martin *et al.* 2009).

Male size distributions (Snelson 1985; Farr *et al.* 1986; Trexler *et al.* 1990; Ptacek & Travis 1996; Seda *et al.* 2012) and morphological traits (Seda 2010; Ptacek 2005) vary substantially within and between populations despite high gene flow (Trexler 1988; Apodaca *et al.* 2013). Size at maturity in males of *P. latipinna* is partially controlled by patrilial inheritance (Travis 1994), where genes on the Y-chromosome (Lampert *et al.* 2010) are thought to influence adult standard length (SL: distance from tip of snout to caudal peduncle; Ptacek & Travis 1996; Seda *et al.* 2012). Environmental factors (e.g., temperature, salinity; Trexler & Travis 1990; Trexler *et al.* 1990), however, are also known to alter male size at maturity through developmental plasticity in length of the juvenile period (Trexler & Travis 1990). Understanding why this life history variation arises and is maintained under local conditions is important, because male size at maturity is correlated with sexual behaviors and mating strategy: larger males erect an enlarged dorsal fin in a courtship display to elicit female cooperation during mating, while smaller males rely primarily on forced copulation attempts (Farr *et al.* 1986; Ptacek & Travis 1996; Seda *et al.* 2012). Further, as males grow minimally after maturation (Snelson 1982; Travis 1994), a small male will never attain the size of a large male during his lifetime. In addition to this body size polymorphism, the size of the dorsal fin in male sailfin mollies is strongly, positively allometric with body size, and its expression is

influenced by both genetic and environmental factors (Ptacek 2002; Hankison & Ptacek 2007; Loveless *et al.* 2009). Females of *P. latipinna* prefer males with the largest lateral projection area (LPA: body area + dorsal and caudal fin areas: Ptacek & Travis 1997; MacLaren *et al.* 2004) making dorsal fin size a clear target of sexual selection.

Therefore, variation in life history and morphology in response to the salinity environment may strongly influence male fitness, hence, contributing to population divergence between habitats in this species.

The primary objectives of this study were to determine the degree of phenotypic plasticity in life history and morphology between salinity environments and at what level (sire size class, family or their interactions with salinity environment) plasticity influences the expression of these traits in males and females of *P. latipinna*. I used sires and dams from a single brackish-water population (ranging in salinity from 3 to 34 ppt) and employed a full-sibling split brood design, rearing juveniles in two different salinity treatments: low salinity (2 ppt salinity) and brackish water (20 ppt salinity). I tested for salinity effects on growth rate and survival in juveniles, size and age at sexual maturity in both sexes, and size of target morphological traits in males is known to be associated with sexual and natural selection. I compared variation among 12 different families using sires from both ends of the natural male size distribution (six small: 20-29 mm SL; six large: 49-57 mm SL) found in this population. I tested the hypothesis that phenotypic plasticity in response to salinity environment produces variation in life history traits (i.e., age, body length (SL), and mass at maturity) and morphological targets of sexual selection (i.e., dorsal fin length). I predicted that greater osmotic stress in low salinity (2 ppt salinity) would result in slower growth, longer development times, and potentially smaller body and dorsal fin ornament size. I discuss my findings in the context of life

history evolution and adaptation to local conditions.

METHODS

Adults of *P. latipinna* were collected from a single brackish water population (Steve's Ditch: N29°58.379', W084°23.357') in Wakulla County, Florida in May and August of 2014 and 2015. This population has been well characterized for its distribution of male sizes (SL) at maturity and rates of mating behaviors (courtship displays, gonopodial thrusts; Ptacek & Travis 1996, Seda *et al.* 2012), and its large variance in male size at maturity (range of SL: 19 mm – 68 mm), which is strongly, positively correlated with courtship display rates (Seda *et al.* 2010). In addition, this saltmarsh site experiences a wide range of salinities (3-34 ppt) seasonally (unpubl. data), thus, fish collected from this population should be able to osmoregulate in both low salinity and saltwater. Fish were collected using a 2.8 x1.2 m seine and a cast net, which is effective for collecting a random sample of females and males of all size classes (Travis & Trexler 1987, Hankison *et al.* 2006; Seda 2010). Fish were transported to Clemson University, Clemson, South Carolina, USA, and housed in the Aquatic Animal Research Laboratory (Animal Research Protocol No. AUP2014-017). Wild-caught males that represented the upper (<25th percentile; >48mm) and lower quartiles (>75th percentile; <30mm) of male size distributions in this population (Seda 2010, Seda *et al.* 2012) were mated to first-generation laboratory-reared virgin females to ensure paternity. This allowed me to compare reaction norms between salinity treatments among families (wild-caught pregnant females may be inseminated by multiple males of varying size: Bisazza 1993; Travis *et al.* 1990; Trexler *et al.* 1997) and to compare variation between salinity

treatments among families sired by small males to those sired by large males. To minimize maternal effects, virgin females were raised in isolation in 3.8 L aquaria that shared brackish water (12 ppt) via a recirculating system. At maturity, virgin females were transferred to 37.85 L aquaria with brackish water (12 ppt) and housed with a male (sire-dam pair). Sire-dam pairs were fed daily with commercial flake food (Ocean Star International Freshwater Flake (60%) with Brine Shrimp Flake (38%) and Spirulina Flake (2%) mixture; Burlingame, California) *ad libitum*. Previous studies have shown that fish reared in these laboratory conditions are indistinguishable in both expression of mating behaviors and morphology from wild-caught fish in several molly species including *P. latipinna* (Ptacek 2002; Ptacek *et al.* 2005, Hankison *et al.* 2006).

Twelve broods were included in this split brood experimental design (6 with sires <30mm and 6 with sires >48mm) and offspring from these broods were reared to sexual maturity between October 2014 and April 2016. Broods with 30-50 individuals were used into the experiment, so starting population density within a tank was standardized at 20 ± 5 individuals. At parturition, broods were transferred to a climate-controlled greenhouse (Biosystems Research Complex Greenhouse; Animal Research Protocol No. AUP2014-053), and placed into a full-sibling split brood design where siblings were divided and reared in one of two salinity treatments (low salinity (2ppt) or brackish (20ppt)) in 568 L Rubbermaid tanks organized in a 4x4 Latin square design (Figure 2.1). Because experimental aquaria were very large, competition between offspring should have been low (Trexler & Travis 1990). The Latin square design helped to minimize other environmental differences (e.g., temperature, light intensity) that may have resulted from room location, but weekly temperature and salinity readings were measured using an YSI Pro30 instrument to assess variability in these environmental variables across

replicate tanks. Due to the logistical constraints of such a large rearing design, broods entered the experiment on different dates (10/2014-9/2015). To account for differences in average photoperiod within and between families, photoperiod data for Clemson, SC was obtained from a US Navy database (<http://aa.usno.navy.mil>). Water temperatures were kept within normal summer conditions ((26°C to 30°C); Trexler *et al.* 1992), which required submersible heaters during the winter months. Each tank was equipped with an individual water pump (flow rate=2460 L/Hr) that provided aeration and biological filtration via a recirculating system. Water was prepared with conditioned tap water using Amquel, Novaqua, Marine Buffer, and Instant Ocean salt. Water quality parameters (pH, ammonia, nitrite) were tested weekly (API test kits) and were always within acceptable ranges. Fish were fed *ad libitum* once daily with ground fish flake food (Ocean Star International Freshwater Flake (60%) with Brine Shrimp Flake (38%) and Spirulina Flake (2%) mixture; Burlingame, California). Partial water changes were performed every three weeks.

Censuses were conducted every three weeks and all fish were measured for standard length ((SL); Ptacek & Travis 1996; Seda *et al.* 2012). Juvenile fish were returned to the experiment; mature fish were removed, euthanized using MS-222, and photographed. Mature males were identified by the complete fusion of the anal fin into an intromittent organ, the gonododium (Cummings 1943), and females were identified by the appearance of a brood spot (Constantz 1989). Broods remained in the experiment until all individuals died or were removed at sexual maturity. In order to control for differential mortality, broods where all individuals died prior to census five, or 105 days, were removed from analyses. This experimental design generated data at the family and individual level. Because it was impossible to identify individuals within a brood, family

averages from each tank and salinity treatment were recorded as the observational unit. It is important to note that I did not replicate family within salinity treatment. However, prior studies have found considerable variation among family genotypes of life histories in response to environmental variation (e.g., Trexler & Travis 1990; Trexler *et al.* 1990), so variation among family in response to salinity is expected.

Average growth, mortality, and maturation rates were recorded for each family/salinity treatment. Because families varied in the duration of time it took for all juveniles to die or reach sexual maturity, linear and polynomial growth models were fitted to average juvenile size (SL) by age (days) for 105 days only (minimum duration of any family). A REML model (Patterson & Thompson 1974) tested the main effect of salinity treatment, as well as potential covariates: average temperature, average photoperiod, and cumulative population density. Family within sire size class and an interaction between family and sire size class were included as random effects. Cumulative population density was measured as the sum of individuals in a tank across all census periods. Survival and maturation rates were assessed across 194 days (9 census periods) using the Kaplan-Meier method and compared using a log-rank chi-square. Families with surviving individuals after 194 days were censored from the dataset.

At the individual level, approximate age (to the nearest three weeks), SL, and wet mass at maturity were recorded and analyzed separately for male and female offspring. None of the three traits were normally distributed, so all were transformed using the natural log to fulfill assumptions of the statistical model. A REML model was used to test the main effects of salinity, sire size class, and their interaction. Average rearing temperature and photoperiod were also included as covariates. To control for family level differences, family within sire size class and an interaction between family and sire

size class were included as random effects. The relationship between natural log-transformed SL and wet body mass was used as a measure of body condition (Anderson & Neumann 1996). A REML model tested for differences due to SL, salinity, and SL by salinity (a measurement of linear slope differences). Average temperature and photoperiod were included as covariates. To control for family level differences, family within sire size class and an interaction between family within sire size class and salinity were included as random effects.

Morphological traits that represent sexually and naturally selected traits of male offspring were measured using ImageJ software: dorsal fin area (male ornament: DFA) and caudal fin area (used in swimming: CFA) were compared to body area (BA). Linear regression between the natural log (ln) transformed trait and natural log (ln) transformed body area (BA) for individuals reared in low salinity and brackish water treatments were performed to calculate adjusted r-square values, slope, and 95% confidence intervals of the slope. A REML model tested for differences due to BA, salinity, and BA by salinity (a measurement of linear slope differences). Average temperature and photoperiod were included as covariates. To control for family level differences, family within sire size class and an interaction between family within sire size class and salinity were included as random effects.

All statistical analyses were performed using JMP Pro version12 (Cary, North Carolina).

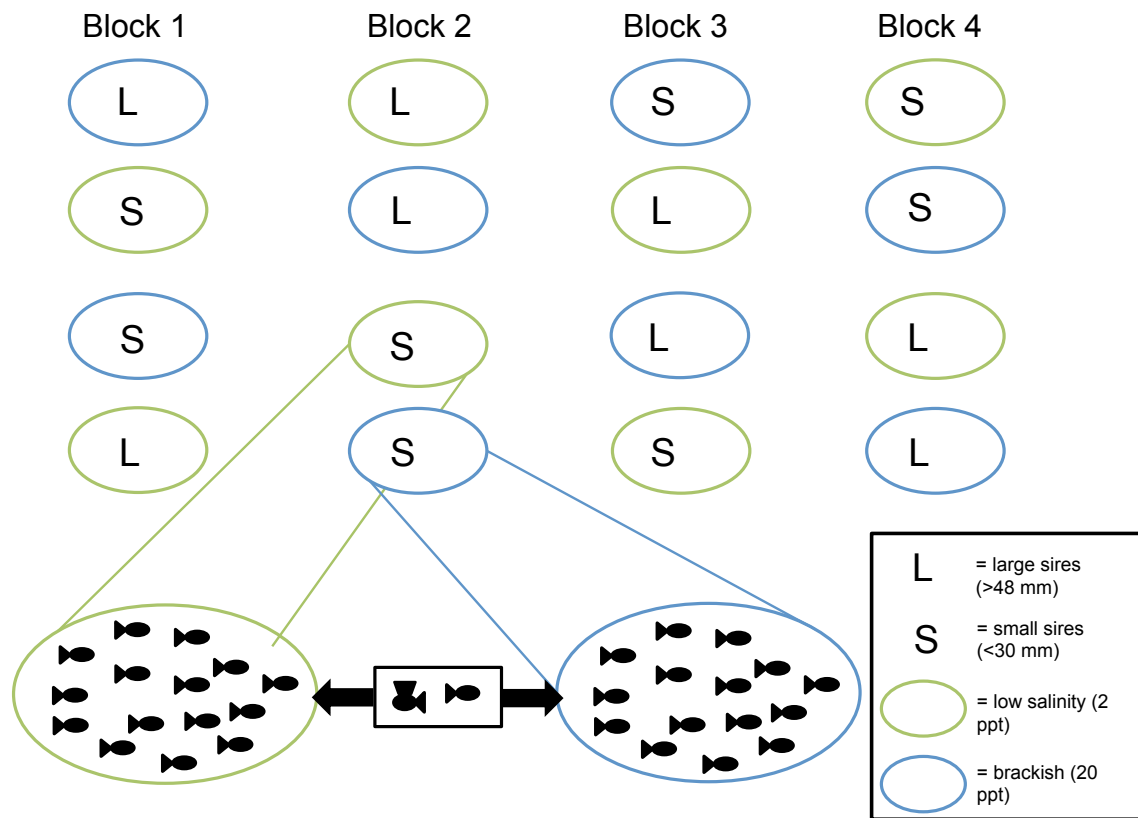


Figure 2.1. Experimental tanks arranged in a 4x4 Latin Square design in the BRC Greenhouse. Each sire size class and salinity combination is represented within a block and occur in every possible row position across blocks.

RESULTS

In total, 309 fish (159 males and 150 females) from 12 families were raised to sexual maturity. Specifically, 72 and 78 females were reared in 2 ppt and 20 ppt, and 72 and 87 males were reared in 2 ppt and 20 ppt, respectively. At least one male and female matured in low salinity and brackish water treatments from every family. Average female age, SL, and mass were greater in low salinity than brackish water treatments (Table 2.1). Male size distributions were skewed towards smaller males in both low salinity and brackish water treatments relative to the observed size distribution of wild-caught males found in Steve's Ditch (Figure 2.2). Summary statistics of life history traits of each family are presented in Appendix I.

The overall survival curves were significantly different between salinity treatments (log-rank chi-square=8.17, df=1, p=0.0043), with mortality rate higher in brackish than in low salinity treatments (Figure 2.3). Total mortality varied substantially among families; some families (e.g., L10) experienced higher mortality in brackish water while others (e.g., L7) had higher mortality in low salinity across both sire size classes (Fig 2.4). Conversely, linear and polynomial growth rates did not differ between salinity treatments (Table 2.2; Figure 2.5). Adjusted r-square values for the linear and polynomial growth curve models were 0.41 and 0.15, respectively (Table 2.2). This lack of fit for both models can be attributed to minimal variation between growth rates across replicate tanks in which the average adjusted r-squares (\pm standard error) for linear and polynomial growth curves were 0.96 (\pm 0.006) and 0.99 (\pm 0.002), respectively. While the AICc values indicated that the polynomial was a better fit than the linear model (Table 2.2), we used both models to determine if growth rates were influenced by salinity,

cumulative density, photoperiod, and temperature. Both linear and polynomial growth models indicated that none of these variables significantly influenced growth rates (Table 2.2; Figure 2.5). All replicate tank averages were pooled to determine an average growth rate of 0.2 mm per day for the first 105 days of the experiment. Finally, female and male maturation rates did not differ significantly across salinity treatments (Female: log-rank chi-square=0.1819, df=1, p=0.6697; Male: log-rank chi-square=0.2807, df=1, p=0.5963; Figure 2.6; Figure 2.7). Nearly 90% of all female and male offspring in the experiment were mature by the ninth census, or 193 days (Figure 2.6; Figure 2.7).

Though females on average took nearly two weeks longer to mature in 2 ppt than 20 ppt (Table 2.1), this difference was not significant in the REML model (Table 2.3). In addition, there was no effect of sire size class or its interaction with salinity on female age at maturity (Table 2.3). Instead, there was considerable variation in family responses to salinity, independent of sire size class (Table 2.3; Figure 2.8). Indeed, family and an interaction between family and salinity accounted for 35% and 48% of the variation in female age at maturity, respectively. Average rearing temperature and photoperiod also significantly modified the age at which females matured (Table 2.3). Increasing temperature resulted in increased age at maturation, whereas increasing photoperiod resulted in decreased age at maturation.

Females in 2 ppt matured at significantly larger sizes (both SL and mass) than their siblings in 20 ppt (Table 2.3). However, sire size class and the interaction between sire size class and salinity did not influence female SL or mass at maturity (Table 2.3). Instead, family response to salinity varied dramatically (Table 2.3; Figure 2.9; Figure 2.10). The main effect of family and its interaction with salinity accounted for 49% and 31% of the variation in female SL and mass at maturity, respectively. This response was

also significantly influenced by average rearing temperature and photoperiod (Table 2.3). Increasing temperature resulted in increased age at maturation, whereas increasing photoperiod resulted in decreased age at maturation.

For males, age, SL, and mass at maturity were not influenced by salinity, sire size class, or their interaction (Table 2.4). However, family within sire size class and an interaction between family within sire size class and salinity significantly influenced all of these life history traits (Table 2.4). Indeed, norms of reaction show that family response to salinity varied dramatically in direction and magnitude (Figure 2.8-2.10) for families sired by both large and small males. Indeed, family differences in response to salinity environment explained between 74 and 77 percent the variation in male age, SL, and mass at maturity. An interaction between family and salinity accounted for between 9 and 12 percent of the variation in these life history traits. Average rearing photoperiod and temperature modified these responses (Table 2.4) as well. Male age, SL, and mass at maturity increased with increasing temperature, but decreased with increasing photoperiod.

Salinity significantly influenced male, but not female, body condition (Table 2.5; Figure 2.11). Specifically, there was a significant interaction between natural log-transformed SL by wet mass and salinity treatment (i.e., the slopes were significantly different) in which the slope between SL and dry mass was steeper for brackish than low salinity treatments. Temperature and photoperiod did not affect male body condition (Table 2.5). For females, there was no interaction between natural log-transformed SL by wet mass and salinity treatment (i.e., the slopes were not significantly different). However, temperature and photoperiod significantly affected female body condition

(Table 2.5). Increasing temperature resulted in increased body condition at maturation, whereas increasing photoperiod resulted in decreased body condition at maturation.

Finally, salinity differences influenced male morphology. Males reared in brackish water had significantly larger dorsal fin areas relative to their siblings reared in low salinity, independent of body size (Table 2.6; Figure 2.12). However, the slopes for the relationship between natural log-transformed dorsal fin area and natural log-transformed body area (BA) did not significantly differ between salinity treatments (Table 2.6; Low Salinity: slope=1.36, 95% CI= 1.17-1.54, $r^2=0.76$; Brackish: slope=1.46, 95% CI=1.33-1.59, $r^2=0.86$). There was also a positive relationship between photoperiod and DFA (Table 2.6), where DFA increased with increasing photoperiod. Conversely, caudal fin area was not significantly different in low salinity and brackish water treatments (Table 2.6; Figure 2.13). The slopes for the relationship between natural log transformed caudal fin area and natural log transformed BA did not significantly differ between salinity treatments either (Table 2.6; Low Salinity: slope=1.03, 95% CI= 0.92-1.14, $r^2=0.83$; Brackish: slope=1.10, 95% CI=1.02-1.17, $r^2=0.91$).

Table 2.1. Summary statistics for age, standard length (SL), and mass at maturity for male and female offspring reared in low salinity and brackish water treatments.

Sex	Life History Trait	Salinity	Minimum	Maximum	Average	Standard Error
Female	Age	Low Salinity	61	336	142.31	7.27
		Brackish	61	296	129.14	7.19
	SL	Low Salinity	26	53	36.89	0.697
		Brackish	22	46	34.37	0.63
	Mass	Low Salinity	0.530	4.940	1.736	0.104
		Brackish	0.503	3.260	1.387	0.075
Male	Age	Low Salinity	43	218	100.54	4.04
		Brackish	42	199	101.55	4.74
	SL	Low Salinity	21	46	30.29	0.61
		Brackish	18	45	30.54	0.69
	Mass	Low Salinity	0.256	2.757	0.890	0.060
		Brackish	0.137	2.786	0.970	0.065

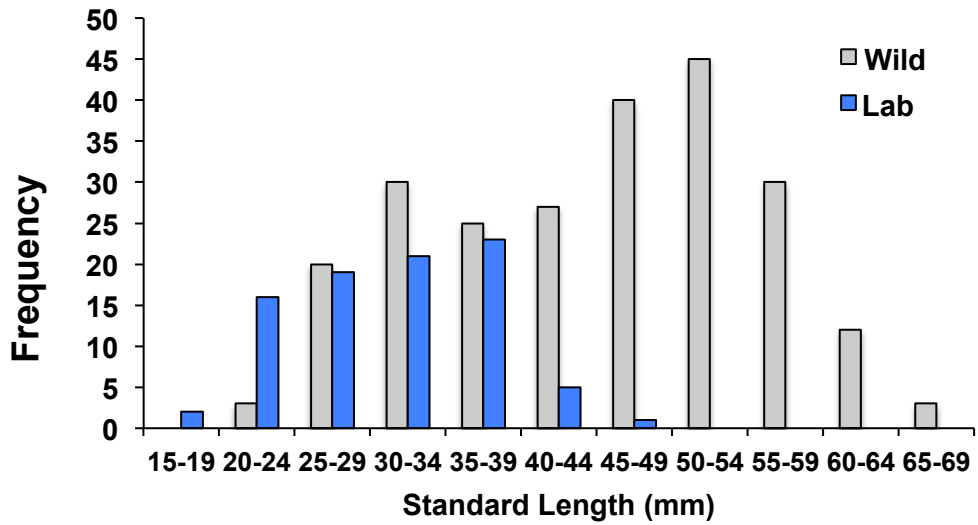
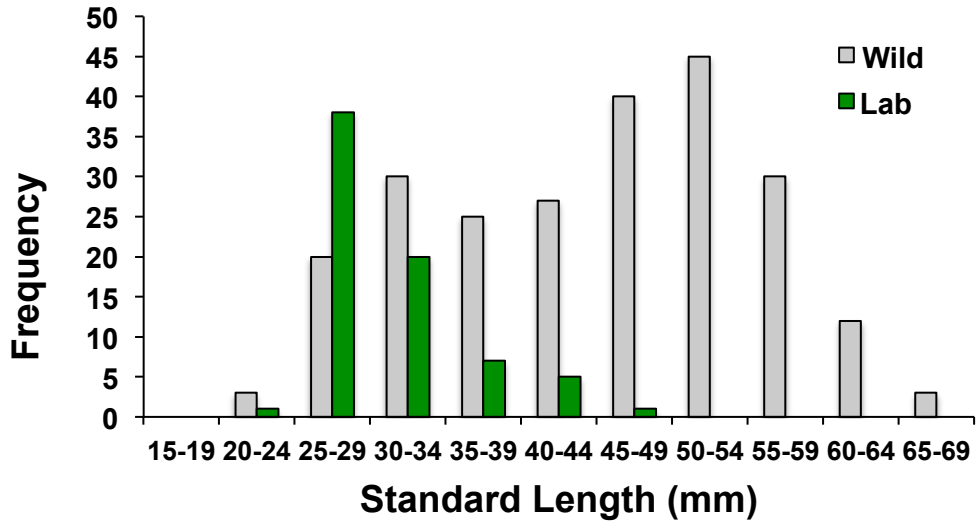


Figure 2.2. Size frequencies of male offspring from low salinity (top) and brackish water (bottom) treatments compared to size frequencies of males frequencies in the native population, Steve's Ditch.

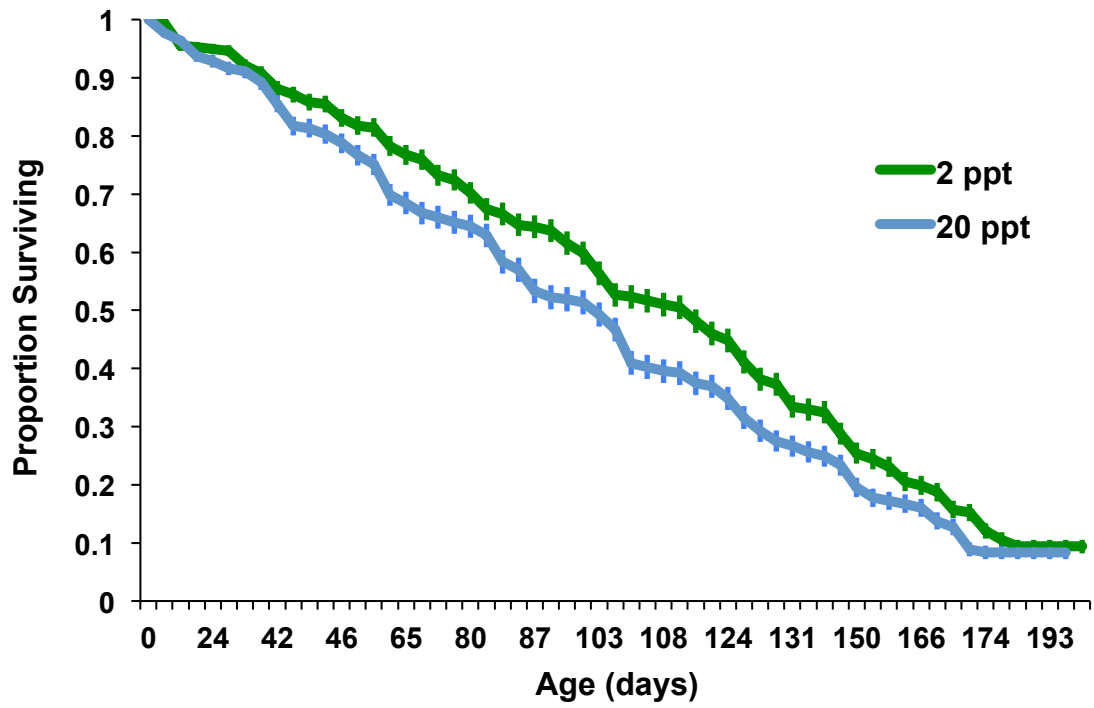


Figure 2.3. Average proportion of surviving juveniles (\pm standard error) in low salinity (green) and brackish water (blue) treatments over 193 days.

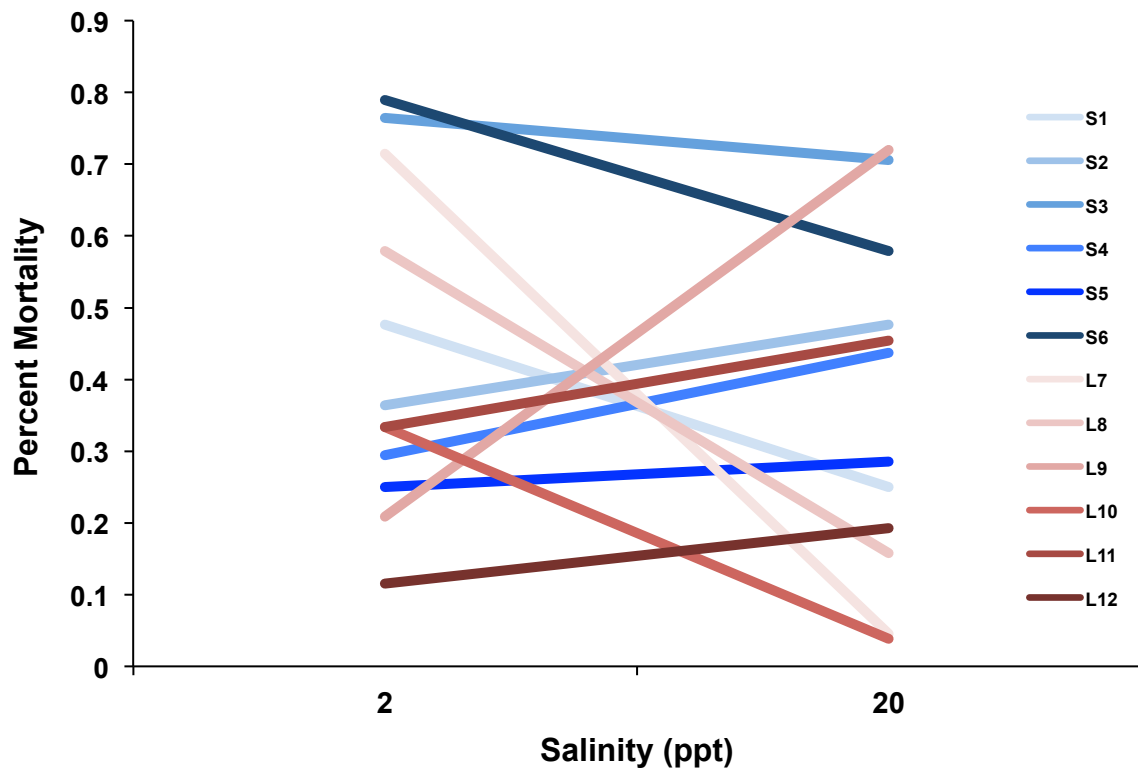


Figure 2.4. Overall percent mortality of offspring reared in low salinity and brackish water treatments across family groups. Blue lines are small sired families (<30mm) and red lines are large sired families (>48mm). Family number represents the rank order of sire sizes (i.e., S1 had the smallest sire at 20 mm and L12 had the largest sire at 57 mm).

Table 2.2. Results of REML analyses testing the effects of salinity, temperature, photoperiod, and cumulative density for growth rate over the first 105 days assuming linear and polynomial models.

Growth Model	Source	DF	F	P	Adj. r²	AICc
Linear	Salinity	1,10	0.0420	0.8415	0.41	73.3
	Temperature	1,12	0.2115	0.6535		
	Photoperiod	1,10	1.5613	0.2403		
	Cumulative Density	1,18	0.3981	0.5357		
Polynomial	Salinity	1,10	0.0041	0.9503	0.15	44.6
	Temperature	1,12	0.0030	0.9571		
	Photoperiod	1,10	0.0551	0.8190		
	Cumulative Density	1,18	0.0108	0.9184		

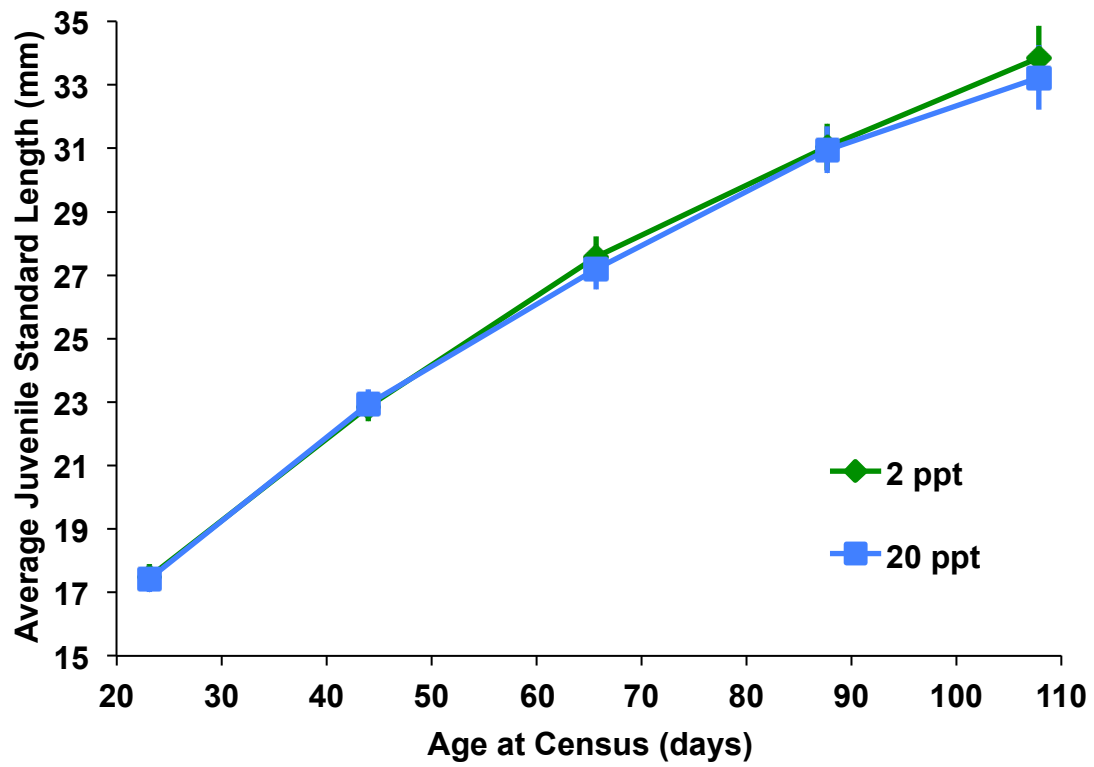


Figure 2.5. Average juvenile standard length (\pm standard error) in low salinity and brackish water treatments for 5 census periods (105 days).

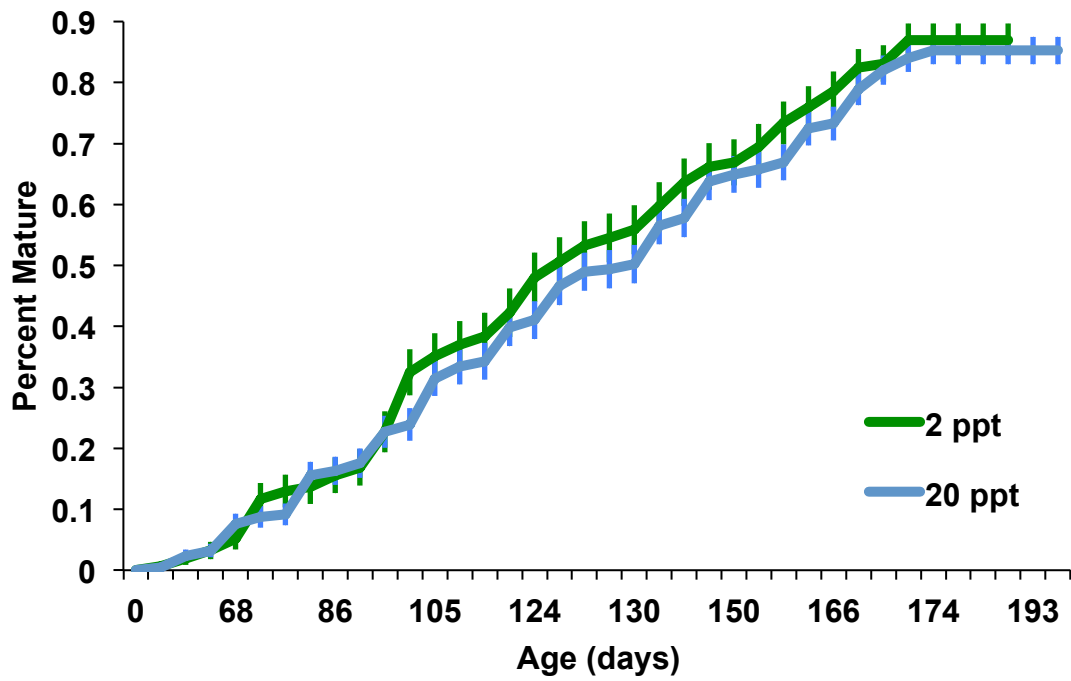


Figure 2.6. Average proportion of females maturing (\pm standard error) in low salinity (green) and brackish water (blue) treatments over 193 days.

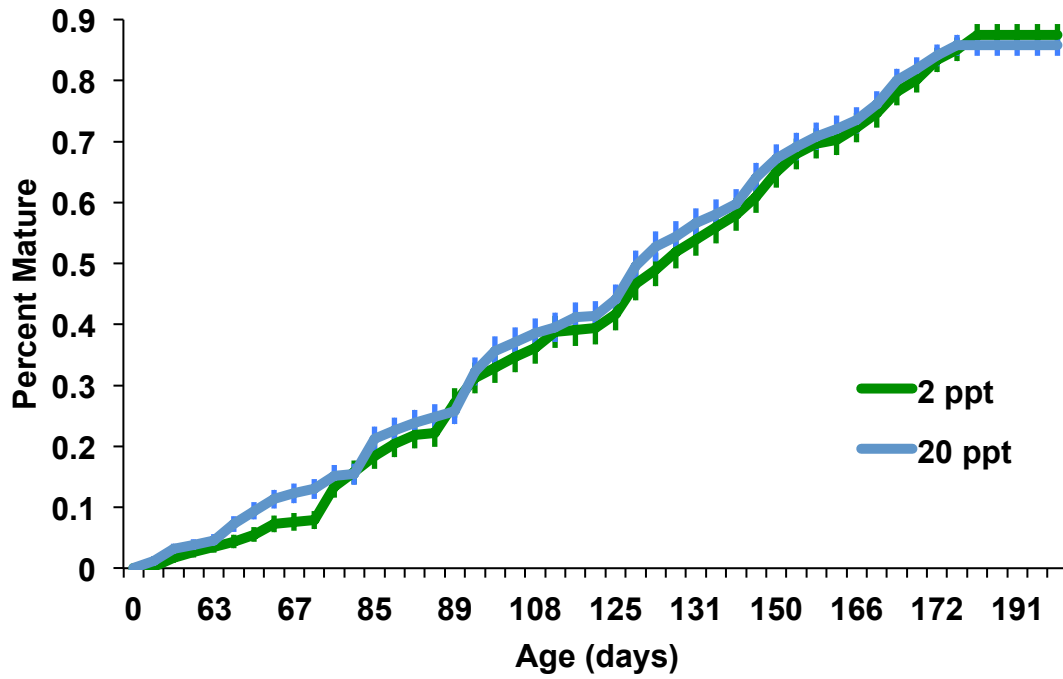


Figure 2.7. Average proportion of males maturing (\pm standard error) in low salinity (green) and brackish water (blue) treatments over 193 days.

Table 2.3. Results of REML analysis for age, standard length (SL), and mass at maturity for female offspring reared in low salinity and brackish water treatments.

Trait	Source	DF	F	P	Adj. r²
Age	Salinity	1,8	2.08	0.1871	0.79
	Sire Size Class	1,8	0.36	0.5629	
	Sire Size Class x Salinity	1,8	1.26	0.2937	
	Temperature	1,138	24.99	<0.0001	
	Photoperiod	1,58	21.83	<0.0001	
	Family ID[Sire Size Class]	10,10	3.95	0.0194	
	Family ID[Sire Size Class] x Salinity	10,124	7.86	<0.0001	
SL	Salinity	1,7	5.72	0.0467	0.77
	Sire Size Class	1,9	0.01	0.9264	
	Sire Size Class x Salinity	1,7	2.40	0.1641	
	Temperature	1,114	6.25	0.0138	
	Photoperiod	1,60	7.06	0.0101	
	Family ID[Sire Size Class]	10,10	5.68	0.005	
	Family ID[Sire Size Class] x Salinity	10,124	5.90	<0.0001	
Mass	Salinity	1,7	5.91	0.0454	0.79
	Sire Size Class	1,9	0.00	0.9835	
	Sire Size Class x Salinity	1,9	2.32	0.1716	
	Temperature	1,117	10.85	0.0013	
	Photoperiod	1,63	10.80	0.0017	
	Family ID[Sire Size Class]	10,10	6.33	0.0033	
	Family ID[Sire Size Class] x Salinity	10,124	5.70	<0.0001	

Table 2.4. Results of REML analysis for age, standard length (SL), and mass at maturity for male offspring reared in low salinity and brackish water treatments.

Trait	Source	DF	F	P	Adj. r ²
Age	Salinity	1,9	1.02	0.3394	0.65
	Sire Size Class	1,7	0.01	0.9377	
	Sire Size Class x Salinity	1,9	1.68	0.2280	
	Temperature	1,122	29.36	<0.0001	
	Photoperiod	1,116	44.75	<0.0001	
	Family ID[Sire Size Class]	10,10	3.33	0.0340	
	Family ID[Sire Size Class] x Salinity	10,133	5.17	<0.0001	
SL	Salinity	1,9	0.68	0.4324	0.57
	Sire Size Class	1,6	0.00	0.9793	
	Sire Size Class x Salinity	1,8	1.67	0.2304	
	Temperature	1,89	22.52	<0.0001	
	Photoperiod	1,92	24.86	<0.0001	
	Family ID[Sire Size Class]	10,10	3.33	0.0334	
	Family ID[Sire Size Class] x Salinity	10,133	3.38	0.0006	
Mass	Salinity	1,9	0.61	0.4571	0.59
	Sire Size Class	1,6	0.01	0.9159	
	Sire Size Class x Salinity	1,9	1.45	0.2611	
	Temperature	1,101	24.68	<0.0001	
	Photoperiod	1,90	25.49	<0.0001	
	Family ID[Sire Size Class]	10,10	3.02	0.0455	
	Family ID[Sire Size Class] x Salinity	10,132	3.88	0.0001	

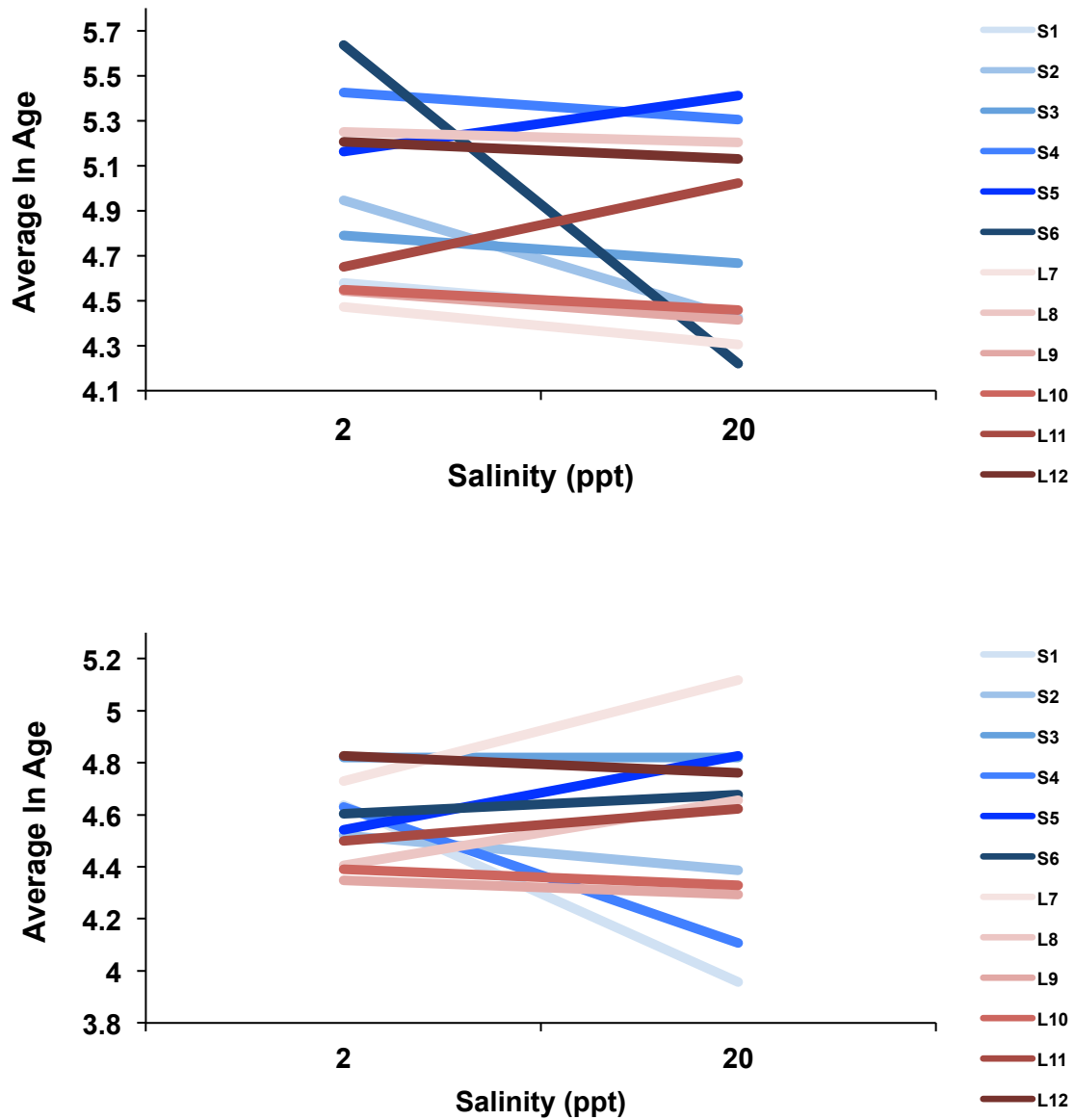


Figure 2.8. Average female (top) and male (bottom) age (transformed by natural log) at maturity for each family reared in low salinity and brackish water treatments. Blue lines are small sired families (<30mm) and red lines are large sired families (>48mm). Family number represents the rank order of sire sizes (i.e., S1 had the smallest sire at 20 mm and L12 had the largest sire at 57 mm).

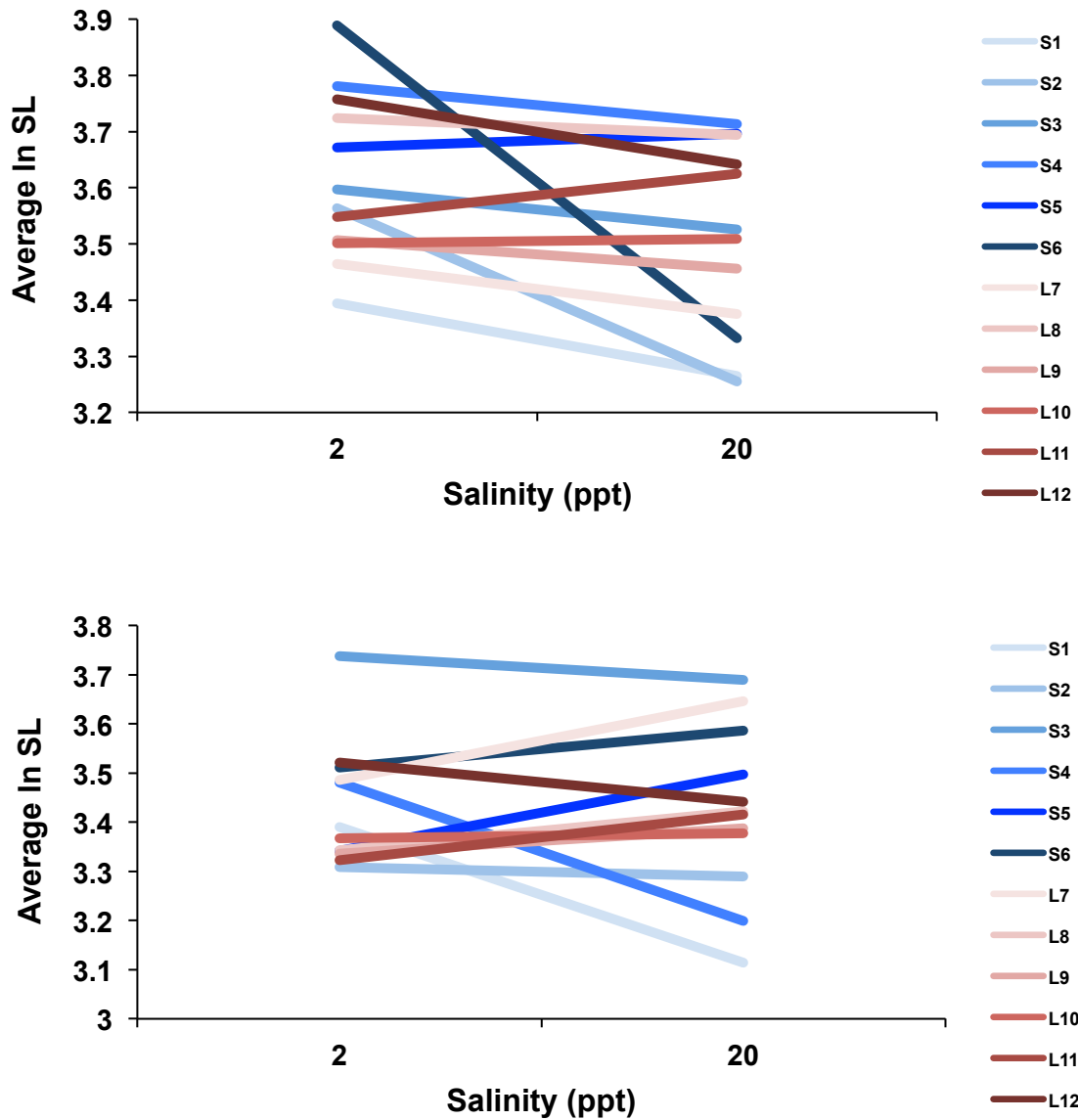


Figure 2.9. Average female (top) and male (bottom) standard length (transformed by natural log) at maturity for each family reared in low salinity and brackish water treatments. Blue lines are small sired families (<30mm) and red lines are large sired families (>48mm). Family number represents the rank order of sire sizes (i.e., S1 had the smallest sire at 20 mm and L12 had the largest sire at 57 mm).

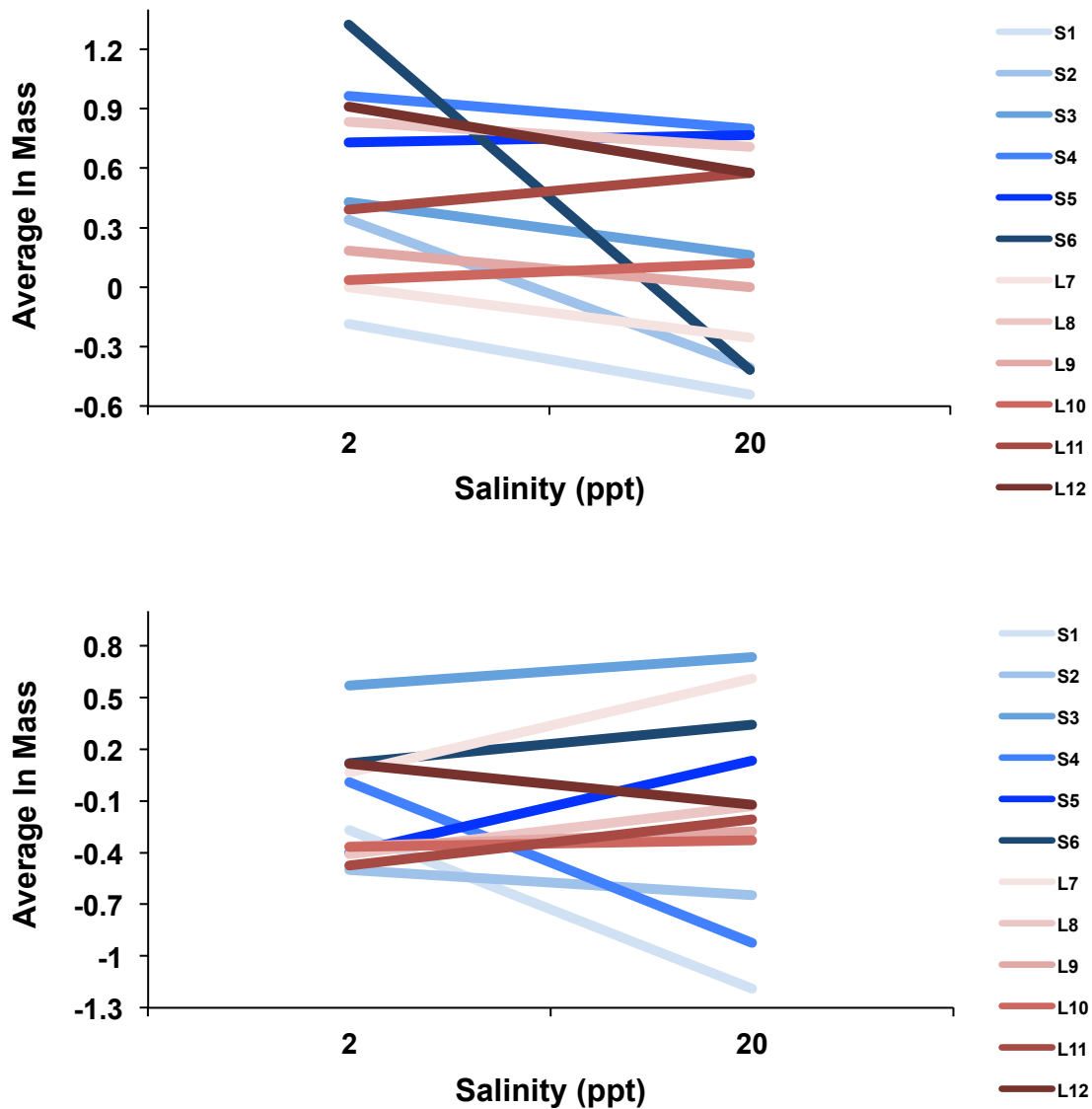


Figure 2.10. Average female (top) and male (bottom) mass (transformed by natural log) at maturity for each family reared in low salinity and brackish water treatments. Blue lines are small sired families (<30mm) and red lines are large sired families (>48mm). Family number represents the rank order of sire sizes (i.e., S1 had the smallest sire at 20 mm and L12 had the largest sire at 57 mm).

Table 2.5. Results of REML analysis comparing natural log-transformed standard length by mass for female and male offspring reared in low salinity and brackish water treatments.

Sex	Source	DF	F	P	Adj. r²
Female	Salinity	1,8	0.09	0.7743	0.97
	ln(SL) x Salinity	1,24	0.11	0.7384	
	ln(SL)	1,64	2245.38	<0.0001	
	Temperature	1,21	5.49	0.0288	
	Photoperiod	1,15	11.71	0.0038	
Male	Salinity	1,5	0.41	0.5529	0.99
	ln(SL) x Salinity	1,119	10.10	0.0019	
	ln(SL)	1,118	6763.65	<0.0001	
	Temperature	1,20	0.10	0.7499	
	Photoperiod	1,19	0.02	0.9028	

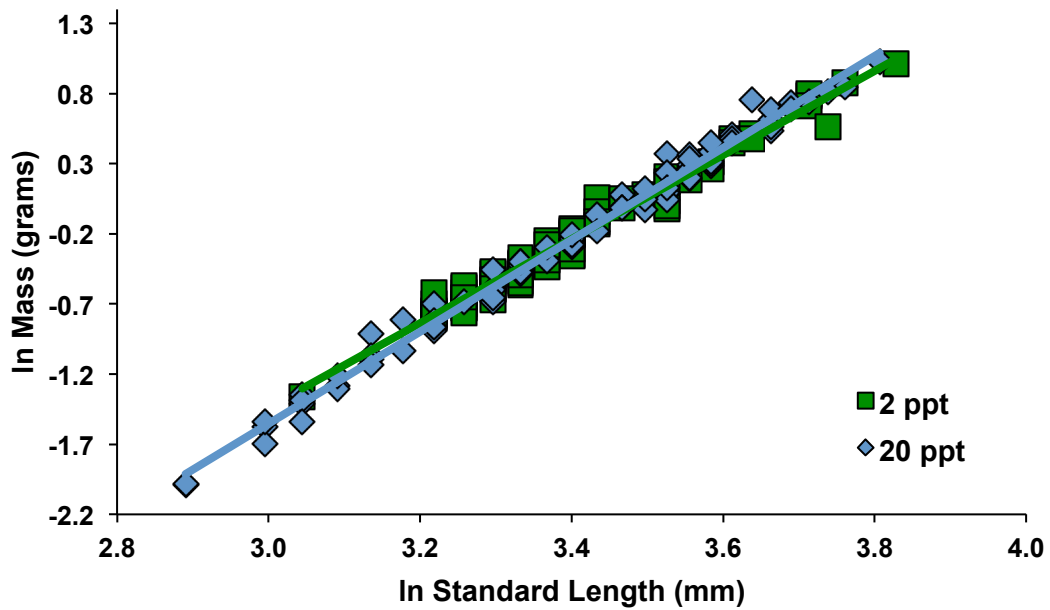
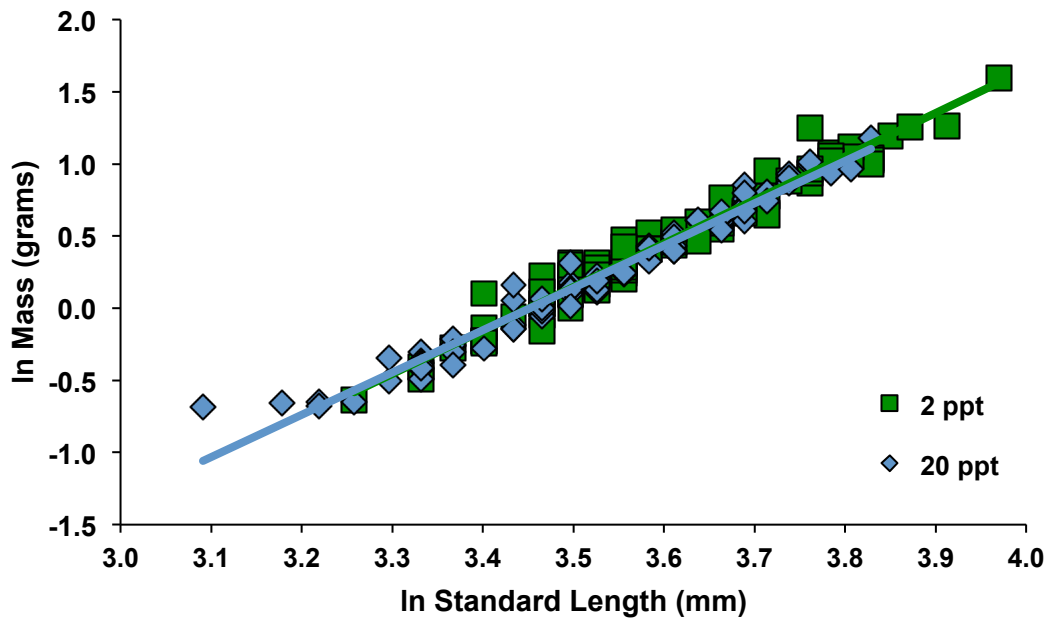


Figure 2.11. The relationship between natural log-transformed standard length (mm) and mass (grams) for females (top) and males (bottom) reared in low salinity and brackish water treatments.

Table 2.6. Results of REML analysis comparing natural log-transformed dorsal fin area (DFA) and caudal fin area (CFA) by body area (BA) of male offspring reared in low salinity and brackish water treatments.

Trait	Source	DF	F	P	Adj. r²
DFA	Salinity	1,5	14.97	0.0133	0.91
	Salinity x ln(BA)	1,115	0.40	0.5293	
	ln(BA)	1,126	601.14	<0.0001	
	Temperature	1,14	0.72	0.4116	
	Photoperiod	1,33	12.34	0.0013	
CFA	Salinity	1,9	0.37	0.5597	0.94
	Salinity x ln(BA)	1,142	0.04	0.8325	
	ln(BA)	1,144	941.44	<0.0001	
	Temperature	1,44	0.34	0.5621	
	Photoperiod	1,31	1.31	0.2611	

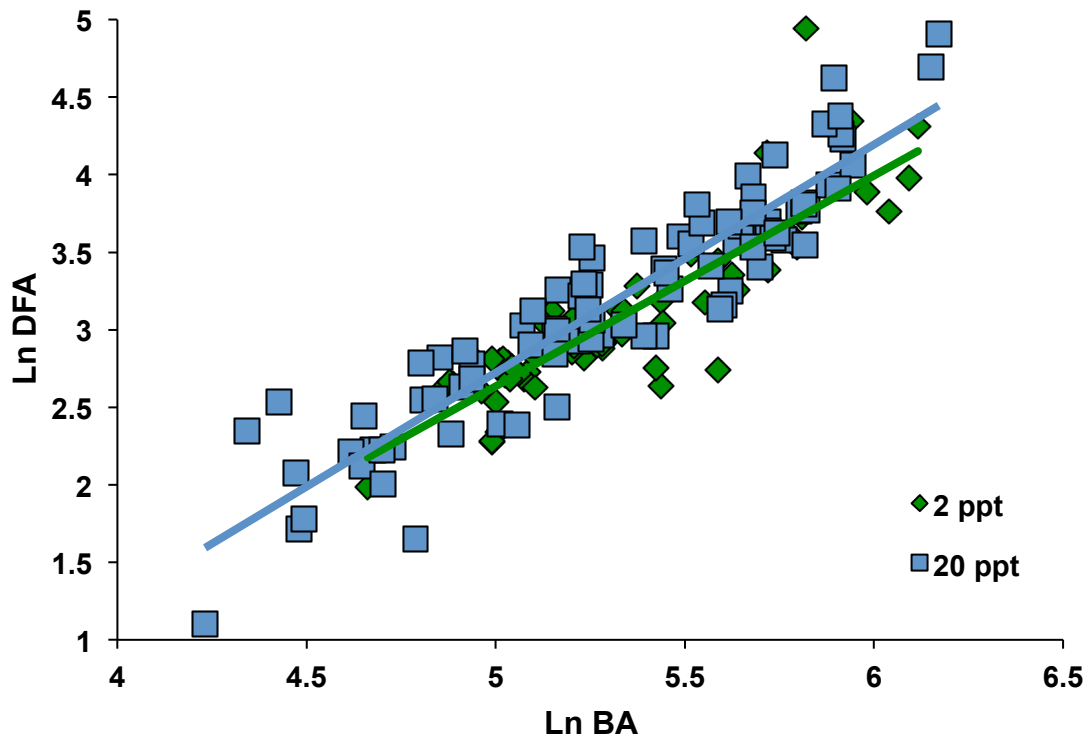


Figure 2.12. The allometric relationship between natural log-transformed dorsal fin area (DFA) and body area (BA) for males reared in low salinity and brackish water treatments.

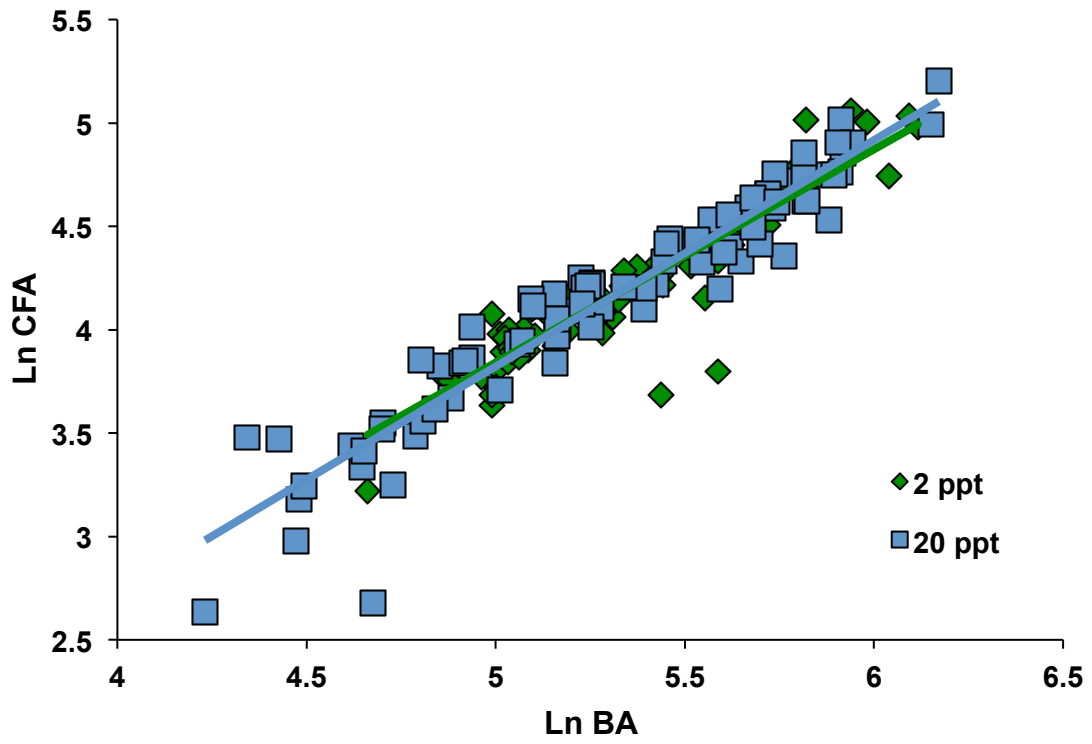


Figure 2.13. The allometric relationship between natural log-transformed caudal fin area (CFA) and body area (BA) for males reared in low salinity and brackish water treatments.

DISCUSSION

Growth and Mortality Rates

Comparisons of growth and mortality rates indicated that *P. latipinna* juveniles perform equally well – if not better – in low salinity conditions than in brackish water conditions. While there were no differences in growth rate between salinity treatments, mortality rate was somewhat higher in the brackish water treatment relative to the low salinity treatment. This finding does not support the prediction that growth rates would shift in response to an environmental stressor (osmoregulation in low salinity), as predicted by life history evolution theory (Sibly & Calow 1989). Juveniles reared in both 2 ppt and 20 ppt had an average growth rate of 0.2 mm SL per day, which is similar to the growth rates reported in other laboratory rearing studies in *P. latipinna* (Snelson 1982; Trexler *et al.* 1990). In addition, a field reciprocal transplant experiment (Trexler & Travis 1990) found that juvenile growth rates in *P. latipinna* varied by season, year, and among family groups. However, when environmental factors were significant, fish grew faster in brackish water ponds (Trexler & Travis 1990). In a corresponding laboratory experiment, Trexler *et al.* (1990) found that juvenile growth rates differed between the sexes: male growth rate did not differ between populations from fresh or brackish water sites and responded minimally to environmental variation, whereas female growth rate was faster in warm (29°C), brackish water (20 ppt) relative to cooler (23°C), fresher water (2 ppt). However, growth rate was influenced much more by temperature than salinity (Trexler *et al.* 1990). In my experiment, replicate tanks were subject to variable photoperiods (i.e., 10-14 hours per day) and temperatures (i.e., 26°C to 30°C), which potentially masked

subtle differences in growth rate in response to salinity environment. In another molly species, *Poecilia vivipara*, Araújo & Monteiro (2013) found that juveniles from a brackish water population grew faster in freshwater than conspecifics from a freshwater population, indicating genetic differentiation may cause differences in growth rates between salinity environments. Taken together with my results, these studies suggest that genotype and environmental factors such as temperature and photoperiod may influence growth rates to a greater degree than does salinity. A limitation of my experimental design was my inability to assess growth rate at the individual level; only measuring family response is possible without uniquely marking individual fish. Therefore, I cannot determine individual variability in growth rate within families, yet such variation provides the raw material through which selection can act on phenotypic plasticity (Mangel & Stamps 2001).

Mortality rates differed in response to salinity environment but not in the direction predicted; mortality rate was somewhat higher in brackish water than in low salinity treatments. Trexler *et al.* (1992) found that in adult *P. latipinna*, survival was higher in brackish water than in freshwater during summer and winter conditions, whereas juvenile survival in fresh and brackish water habitats was temporally inconsistent. However, fish were more likely to survive to maturity in freshwater than brackish water in one year of the experiment (Trexler *et al.* 1992). Bachmann & Rand (2009) observed increased mortality of juvenile *P. latipinna* in freshwater (0 ppt) relative to brackish water (15 ppt), but survival was equally high in 2 ppt and 15 ppt. One possible explanation for higher mortality in the 20 ppt treatment may be that our low salinity treatment (2 ppt) did not have imposed a significant osmotic stress. Another explanation may be as a result of life history trade-offs. Juveniles reared in brackish water may have been allocating

energy resources towards gonadal development at the expense of maintenance and growth (e.g., females in many families matured earlier in brackish water), although overall rates of maturation did not differ between salinity treatments. Indeed, Gadgil & Bossert (1970) hypothesized that devoting energy towards reproduction results in decreased growth and survivorship. Such a trade-off between reproductive effort and somatic condition has been observed in other female livebearers (*Poecilia reticulata*: Reznick 1983; *Gambusia holbrooki*: Alcaraz & García-Berthou 2007). Wild-caught females of *P. latipinna* had similar body conditions in fresh and brackish water habitats, but those from freshwater habitats exhibited reduced reproductive allotment and fecundity (Martin *et al.* 2009). Therefore, it is possible that fish in the low salinity treatment suffered lower mortality because they allocated more energy towards somatic maintenance instead of gonadal development. Future studies should explore the potential trade-offs between somatic maintenance and reproductive effort at the onset of maturation in male and female *P. latipinna* across a range of environmentally relevant salinities.

Male and Female Comparison

Salinity significantly affected female, but not male, life history traits. On average, females were older at maturity in 2 ppt than 20 ppt, but this difference was not significant (Table 2.1; Table 2.8). Females were significantly larger (SL and mass) at maturity in low salinity than brackish water treatments. Delayed maturation (Zimmerer 1983; Trexler & Travis 1990; Trexler *et al.* 1990) and larger body size (Trexler *et al.* 1990) in low salinity is consistent with previous findings. Sire size class and an interaction between sire size

class and salinity did not significantly impact any of the three female life history traits examined. Instead, family variation within sire size class and an interaction between family and salinity explained at least 80% of the total variation in female life history traits. In addition, differences in average rearing temperature and photoperiod were more important determinants of female life history traits than salinity. I observed a negative relationship between photoperiod and age, size, and mass at maturity for females. This is consistent with the finding that female *P. latipinna* cease ovary development somewhere between 12 and 14 hours of daylength, regardless of temperature (Grier 1973). Conversely, I observed a positive relationship between temperature and age, size, and mass at maturity for females, even though temperature variation was minimal and was always within normal summer conditions ((26°C to 30°C); Trexler *et al.* 1992). Trexler *et al.* (1990) also found that temperature effects far exceeded salinity effects in determining female age at maturity. Trexler *et al.* (1990) also found that female size was less responsive to environmental factors than female age at maturity. I found a similar pattern: female age at maturity was largely determined by an interaction between family and salinity (48% of the variation in the data), whereas family alone accounted for nearly 50% of the variation in female SL and mass at maturity. Taken together, female age at maturity may be more phenotypically plastic than size at maturity. Further, differences between families suggest female size at maturity has a genetic component. It is possible that maternal effects are responsible for these differences. However, maternal effects should have been minimized in my experiment because dams were laboratory-reared, and experienced similar environmental conditions during ontogeny.

Salinity did not significantly impact male life history traits. Sire size class and an interaction between sire size class and salinity also did not impact any of the three life

history traits examined. Instead, family within sire size class accounted for as much as 77% of the variation in life histories, and an interaction between family and salinity accounted for as much as 12%. This means that family was more than twice as important in determining male age, than female age at maturity. Male SL and mass at maturity were also less responsive to an interaction between family and salinity than were female SL and mass, suggesting that male life history traits are less phenotypically plastic than female life histories. This result is congruent with Trexler *et al.* (1990) where over 50% of the variation in female age at maturity was attributed to environmental factors, whereas less than 12% of the variation in male age at maturity could be explained by temperature and salinity conditions. It has been suggested that male size is inherited at least in part, through genes on the Y-chromosome because males do mature at similar sizes as their sires under certain laboratory conditions (Travis 1994). My results however, did not show a strong effect of sire size class, independent of family differences in male offspring SL at maturity. For instance, large-sired families did not produce any large sons (>48mm), and male size distributions in both salinity treatments were smaller than the male size frequencies that are characteristic of their native population (Figure 2.2). Trexler *et al.* (1990) also raised males that were smaller on average than those collected from brackish populations. They suggested this pattern could have occurred if frequencies of male maturation genotypes changed between the sampling period and the laboratory study (Trexler *et al.* 1990). Because I controlled for sire size, this hypothesis is not sufficient to explain my results. Instead, male life history traits may exhibit phenotypic plasticity in response to environmental conditions that were not captured in my laboratory study. For example, the social environment is an important determinant of male size in many poeciliid fishes (e.g., platyfish: Borowsky 1978;

swordtails: Walling *et al.* 2007; guppies: Campton & Gall 1988, Magellan & Magurran 2009; sailfin mollies: unpublished data), where juvenile males generally delay maturation in the presence of adult males. This hypothesis requires that male life histories are phenotypically plastic, and that differences in salinity may not have imposed enough of a stressor to cause physiological trade-offs in male *P. latipinna*, at least in this experiment.

While males were relatively insensitive to salinity environment, temperature and photoperiod differences significantly impacted male life history traits. This result is consistent with findings that temperature is more important than salinity in determining male age, SL, and mass (Trexler *et al.* 1990) and testis biomass at maturity (McManus & Travis 1998). The effects of photoperiod and temperature are correlated and often synergistic (Boeuf & Le Bail 1999). Interestingly, I found that small increases in temperature (26°C to 30°C) resulted in larger male body size, while longer day length resulted in earlier maturation at smaller body size at maturity. Longer day length stimulates food intake and food conversion efficiency in many fishes (Boeuf & Le Bail 1999), and increases gonadosomatic index in some poeciliid fishes (*Poeciliopsis gracilis*; *P. sphenops*: Burns 1985). I did not systematically vary photoperiod; instead, photoperiod differences resulted as a side effect of conducting the experiment in a greenhouse over the course of 1.5 years. To my knowledge, the influence of photoperiod on male life history traits in *P. latipinna* is not well understood, even though photoperiod is arguably the most predictable environmental cue (Migaud *et al.* 2010). Coordinating reproductive efforts with reliable cues can optimize allocation of energy resources (Migaud *et al.* 2010), and may be especially important in highly variable environments (Robinson *et al.* 2011). Snelson (1984) found that peak reproduction occurs in spring and fall months in *P. latipinna* populations. Consequently, selection to coordinate

gonadal development in response to changing photoperiod in *P. latipinna* should be strong. Future studies should investigate the influence of photoperiod on male maturation in *P. latipinna*, as it may play a role in maintaining seasonal variation of male size distributions.

Male, but not female, body condition was significantly affected by salinity treatment where the relationship between SL and wet mass increased more rapidly for males reared in brackish water relative to the low salinity treatment. Female body condition was not influenced by salinity. Instead, there was a slight positive and negative relationship between female body condition and temperature and photoperiod, respectively. This result is consistent with the findings of Martin *et al.* (2009) in which field surveys of *P. latipinna* across marshes varying in salinity revealed that male body condition was better in brackish water than freshwater marshes, whereas female body condition did not vary in response to salinity. Instead, female reproductive allotment in terms of gonadosomatic index and fecundity was reduced in freshwater than brackish water marshes (Martin *et al.* 2009). My results coupled with those of Martin *et al.* (2009) suggest that male and female sailfin mollies may exhibit different patterns of life history trade-offs. While female age and size at maturity shift in response to salinity environment, male life histories are more constrained by family genotype. However, male body condition is reduced in low salinity than brackish water, suggesting that males undergo physiological trade-offs in response to salinity environment.

MALE MORPHOLOGY

As predicted, the sexual ornament (dorsal fin area) was sensitive to osmotic stress, while a trait more likely subject to natural selection (caudal fin area) was not influenced by salinity environment. The dorsal fin shows positive allometry with body size in males (Ptacek 2002; Hankison & Ptacek 2007) as a result of strong sexual selection for increased lateral projection area (LPA: sum of body and dorsal and caudal fin areas; Ptacek & Travis 1997; MacLaren *et al.* 2004; Hankison *et al.* 2006). The handicap hypothesis predicts that female preferences for elaborate sexual ornaments have evolved because such traits are condition-dependent, and therefore, honest signals of mate quality (reviewed in Andersson 1994). The theory has been widely investigated across taxa, but empirical support remains sparse (Cotton *et al.* 2004). Emlen *et al.* (2012) demonstrated that ornamental horn size in the rhinoceros beetle (*Trypoxylus dichotomus*) is sensitive to nutritional quality via the insulin/insulin like growth factor pathway. In livebearers, there is evidence that diet influences secondary sexual characters in males (*P. reticulata*: Hughes *et al.* 2005; Rahman *et al.* 2013; *P. latipinna*: Lange 2013), and that females prefer well-fed males (*P. reticulata*: Kodric-Brown 1989; Grether 2000; *P. mexicana*: Plath *et al.* 2005). Gomes & Monteiro (2008) found morphological divergence among *P. vivipara* populations that inhabited a salinity gradient; however, they concluded that differences were likely attributed to predation pressures instead of salinity. Here I provide preliminary evidence that osmotic stress compromises the size of the ornamental dorsal fin in male *P. latipinna*. Future studies should identify the mechanism responsible for the development of the ornamental dorsal

fin. It would also be informative to investigate the allometric scaling of the dorsal fin area in response to a range of salinities that are environmentally relevant (Cotton *et al.* 2004).

The condition-dependence of sexual traits should be compared to suitable non-sexual traits (Cotton *et al.* 1994). Therefore, I also measured caudal fin areas of individuals reared in low salinity and brackish water treatments. The caudal fin is involved in thrust generation and maneuverability, and therefore is more likely subject to natural selection in addition to sexual selection (Hankison *et al.* 2006; Langerhans *et al.* 2004). For example, mosquitofish (*Gambusia affinis*) had larger caudal fins in predator populations relative to predator-free populations (Langerhans *et al.* 2004). Gomes & Monteiro (2008) found the same pattern in *P. vivipara* in a similar predator regime. Therefore, the caudal fin represents an appropriate non-sexual comparison to the secondary sexual character of the dorsal fin used in the courtship display signal. The prediction that male caudal fin area would be relatively insensitive to salinity environment was supported. Therefore, I can conclude that low salinity compromised the size of a sexually, but not naturally selected trait. This result suggests that the enlarged dorsal fin is condition-dependent in *P. latipinna* and as such, may act as an indicator trait of male quality in this species.

SIGNIFICANCE

Field surveys (Martin *et al.* 2009) and laboratory experiments (Evans 1973, 1975; Nordlie *et al.* 1992) suggest that individuals of *P. latipinna* are physiologically stressed in freshwater relative to brackish water due to the increased demands of osmoregulation. Concordantly, *P. latipinna* exhibits interpopulation size and morphological variation

between salinity environments (Snelson 1985; Farr *et al.* 1986; Trexler *et al.* 1990; Ptacek & Travis 1996; Seda *et al.* 2012) despite high gene flow (Trexler 1988; Apodaca *et al.* 2013). Physiological trade-offs between growth, maintenance, storage, and reproduction via phenotypic plasticity may provide an explanation for this interpopulation variation in *P. latipinna*. Phenotypic plasticity mitigates the effects of environmental stress by allowing organisms to shift their developmental trajectories to maximize fitness in a given environment (Pigliucci 2001). In highly variable habitats, such as coastal estuaries, phenotypic plasticity should be maintained. Indeed, Bamber & Henderson (1999) suggest that selection for plasticity in estuarine fish has pre-adapted them to invading stressful freshwater habitats. Consistent with these predictions, I have shown that *P. latipinna* from a brackish water habitat performed equally well – if not better – in a low salinity habitat relative to a brackish water habitat in terms of growth and mortality rates. I found that females responded consistently to salinity environment, where females were smaller at maturity in brackish water relative to low salinity treatments. Males also responded plastically to salinity environment; though, the direction and magnitude depended largely on family genotype. However, the effects of temperature and photoperiod far exceeded the importance of salinity in determining both male and female life histories. These results suggest that salinity variation alone is not sufficient to explain the interpopulation differences in male size distributions consistently observed in *P. latipinna* (Snelson 1985; Farr *et al.* 1986; Trexler *et al.* 1990; Ptacek & Travis 1996; Seda *et al.* 2012). Freshwater and brackish marshes likely vary in additional abiotic factors such as food resources, as well as biotic factors such as predation regime, competition, and community structure. Indeed, Trexler *et al.* (1990) suggested that habitats along salinity gradients are likely subject to differential selection pressures and

survival as salinity changes. All of these variables will need to be assessed before drawing firm conclusions about the factors that influence life history and mating signal variation among populations of *P. latipinna* in different environments.

My results do however, provide support for the prediction that stressful environmental conditions lead to physiological trade-offs. Interestingly, male and female *P. latipinna* exhibited different life history trade-offs in response to salinity environment. Female body size was smaller in brackish water compared to low salinity treatments. Male life history traits, however, did not respond to environmental variation in the salinity environment, but body condition and the sexual ornament (dorsal fin) were compromised, potentially in response to osmotic stress. As a result, it is possible that the strength of sexual selection differs between populations of *P. latipinna* along salinity gradients. Polymorphism in male size and size-associated mating strategy in *P. latipinna* has been proposed to be maintained by a balance between natural selection favoring smaller size through earlier maturation and sexual selection favoring larger size through female mating preferences for increased LPA in males (Travis 1994; Ptacek and Travis 1997; Ptacek 2005). If salinity or other abiotic variables shift the fulcrum of this balance, such as weaker sexual selection in freshwater environments, then variability in male life history and mating strategy could be maintained. Future studies should compare the strength of female mating preferences for large male body size in habitats that vary in salinity.

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APPENDICES

Appendix A. Summary statistics for female and male age, standard length, and mass across all families.

Females			Age					Standard Length				Weight			
Family	Sire SL	Salinity (ppt)	N	Min	Max	Average	St. Error	Min	Max	Average	St. Error	Min	Max	Average	St. Error
S1	20	2	7	64	126	101.71	11.62	26	32	29.86	0.83	0.530	1.250	0.860	0.091
		20	5	64	139	88.80	15.73	25	28	26.20	0.58	0.506	0.707	0.588	0.044
S2	20	2	6	126	149	141.00	3.59	30	41	35.50	1.65	1.106	1.905	1.434	0.126
		20	3	65	136	88.67	23.67	22	33	26.33	3.38	0.503	1.133	0.719	0.207
S3	25	2	3	61	276	146.67	65.79	30	45	37.00	4.36	0.785	3.056	1.784	0.670
		20	4	61	277	128.00	50.04	28	45	34.50	3.66	0.673	2.628	1.345	0.436
S4	25	2	4	191	276	229.25	17.76	40	50	44.00	2.16	1.937	3.536	2.689	0.341
		20	3	166	296	209.33	43.33	40	42	41.00	0.58	1.967	2.474	2.226	0.147
S5	27	2	8	88	253	184.88	21.83	34	44	39.50	1.46	1.258	3.492	2.194	0.276
		20	6	193	235	224.50	7.17	39	43	40.33	0.61	1.720	2.750	2.179	0.144
S6	28	2	2	234	336	285.00	51.00	45	53	49.00	4.00	2.865	4.940	3.902	1.037
		20	1	68	68	68.00	0.00	28	28	28.00	0.00	0.659	0.659	0.659	0.000
L7	49	2	4	68	131	91.00	14.98	28	39	32.25	2.50	0.613	1.727	1.084	0.252
		20	14	68	111	75.21	3.92	27	34	29.29	0.51	0.604	1.206	0.795	0.052
L8	50	2	2	153	237	195.00	42.00	39	44	41.50	2.50	1.804	2.938	2.371	0.567
		20	9	131	237	187.89	16.61	36	46	40.33	1.14	1.492	3.260	2.103	0.204
L9	53	2	14	84	105	94.50	2.91	30	35	33.36	0.34	0.873	1.367	1.211	0.038
		20	4	63	105	84.00	8.57	29	34	31.75	1.11	0.770	1.254	1.022	0.119
L10	54	2	6	85	105	95.00	4.47	32	35	33.17	0.48	0.851	1.223	1.044	0.055
		20	14	85	105	86.43	1.43	31	39	33.50	0.60	0.867	1.753	1.147	0.061
L11	57	2	4	90	110	105.00	5.00	33	36	34.75	0.63	1.308	1.687	1.487	0.092
		20	4	110	230	160.25	30.08	33	43	37.75	2.29	1.275	2.696	1.865	0.336
L12	57	2	12	126	211	184.58	7.90	36	48	43.00	1.13	1.510	3.504	2.553	0.167
		20	11	126	211	170.64	7.31	36	40	38.18	0.40	1.486	2.048	1.787	0.056

Appendix A. continued.

Family	Males			Age				Standard Length				Weight				
	Sire	SL	Salinity (ppt)	N	Min	Max	Average	St. Error	Min	Max	Average	St. Error	Min	Max	Average	St. Error
S1	20	2		4	43	166	115.25	25.86	21	34	30.25	3.12	0.256	1.230	0.889	0.216
		20		10	43	137	56.60	9.35	18	35	22.90	1.53	0.137	1.438	0.389	0.121
S2	20	2		8	92	92	92.00	0.00	25	30	27.38	0.53	0.538	0.732	0.610	0.024
		20		8	51	126	84.25	9.39	20	35	27.25	1.80	0.183	1.287	0.619	0.126
S3	25	2		1	124	124	124.00	0.00	42	42	42.00	0.00	1.765	1.765	1.765	0.000
		20		1	124	124	124.00	0.00	40	40	40.00	0.00	2.080	2.080	2.080	0.000
S4	25	2		8	49	166	107.88	12.08	25	38	32.75	1.52	0.459	1.675	1.092	0.166
		20		6	42	94	64.50	9.99	18	35	25.33	2.99	0.138	1.215	0.554	0.199
S5	27	2		7	85	101	94.14	3.23	26	30	28.29	0.57	0.512	0.848	0.682	0.053
		20		9	83	199	133.89	17.04	27	40	33.33	1.63	0.635	2.005	1.253	0.184
S6	28	2		2	89	112	100.50	11.50	32	35	33.50	1.50	1.050	1.206	1.128	0.078
		20		7	84	112	108.00	4.00	34	39	36.14	0.59	1.263	1.763	1.417	0.069
L7	49	2		2	75	171	123.00	48.00	26	41	33.50	7.50	0.518	2.194	1.356	0.838
		20		7	138	195	168.43	8.84	32	45	38.57	1.85	1.083	2.786	1.927	0.229
L8	50	2		6	74	136	84.33	10.33	25	36	28.50	1.61	0.452	1.365	0.711	0.134
		20		7	74	150	110.86	14.41	24	41	31.29	2.63	0.445	2.090	1.058	0.261
L9	53	2		5	63	91	78.40	6.42	25	31	28.20	1.16	0.481	1.063	0.726	0.109
		20		3	63	84	73.67	6.06	28	33	29.67	1.67	0.670	0.968	0.770	0.099
L10	54	2		8	65	105	81.50	4.56	25	33	29.13	1.03	0.449	1.078	0.725	0.082
		20		11	55	91	77.36	4.70	23	34	29.55	1.15	0.402	1.095	0.765	0.080
L11	57	2		10	81	152	91.70	6.85	25	37	27.90	1.09	0.459	1.611	0.668	0.107
		20		8	81	170	104.88	10.47	27	33	30.50	0.82	0.504	1.133	0.842	0.081
L12	57	2		11	78	218	134.73	16.05	25	46	34.64	2.34	0.468	2.757	1.385	0.260
		20		10	76	198	125.70	15.62	24	39	31.80	1.95	0.355	1.703	1.059	0.183