AN INTEGRATIVE APPROACH TO THE ECOLOGY AND EVOLUTION OF ALTERNATIVE REPRODUCTIVE TACTICS IN MALE *POECILIA LATIPINNA*

by

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DEDICATION

I dedicate this work to my paternal grandfather, Myun Young Kim, and grandmother, Kyung Ja Kim. Their love and support have not gone unnoticed. If they had lived to see this day, they would have been undoubtedly proud.

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ABSTRACT

Understanding the sources of variation that make up complex phenotypes has been a long-standing goal of evolutionary biology. Sexually-selected polymorphisms such as those found in alternative reproductive tactics (ARTs) are an example of complex phenotypes that show extreme variation among multiple traits. Males often show phenotypic traits that vary in size, ornamentation, coloration, and behavior. For my dissertation, I used an integrative approach to investigate aspects of the social, hormonal and genetic effects that contribute to alternative reproductive tactics using male sailfin mollies, *Poecilia latipinna*, a live-bearing fish species. Complex phenotypes have both genetic and environmental sources of variation, and hormones often mediate the interaction between these two sources. Maternal effects and the presence of rival males are two such social environmental factors that can affect male phenotypes. First, I examined the effects of other rival males on male mate choice for conspecific females, and on the changes in circulating levels of the androgen 11-ketotestosterone (KT) and cortisol within a mate choice context. Although rival males did not affect male mate choice, these potential competitors did affect the KT release rates of focal males and females. Further, males released more KT with increasing size of the rival male. Then, I investigated how cortisol release rates varied in female sailfin mollies during gestation to identify the potential effects of maternal stress on son phenotype. I conducted an adrenocorticotropic hormone (ACTH)-challenge to determine the natural range of cortisol by gestating and non-gestating females and whether within and among individual

variation (repeatability) contributed to female cortisol release rates. Gestational status did not correlate with cortisol but females showed high repeatability in cortisol release rates which suggests that variation in maternal stress may affect offspring and that cortisol release rates may be heritable. Lastly, I identified the genetic basis of male phenotypic variation using a genome-wide association study. Using a Bayesian sparse linear mixed model approach, I characterize the underlying genomic architecture of relevant morphological traits that define ARTs in male sailfin such as body size. I found that the phenotypic traits associated with ARTs in male sailfin mollies show a complex genetic architecture with many loci of small effect, which suggests that these ART traits are polygenic and reflect high heritability. Together, these chapters provide greater understanding of the genetic and physiological mechanisms for the variation in male phenotypes of sailfin mollies.

I. INTRODUCTION

Understanding the sources of variation that make up complex phenotypes has been a long-standing goal of evolutionary biology. Sexually-selected polymorphisms such as those found in alternative reproductive tactics (ARTs) are an example of complex phenotypes that show extreme variation among multiple traits.

Alternative reproductive tactics

Sexually-selected traits including vocalizations, weaponry, coloration, and courtship displays, are some of the most impressive ornamental traits among males of many species (Andersson 1994). Some species will show extreme variation among males in these sexually-selected traits and intense competition for mates may result in the evolution of alternative mating tactics. Alternative reproductive tactics (ARTs) are a discrete suite of traits within one sex that are used by individuals as alternate ways to gain access to mates (Oliveira et al. 2008b). Intrasexual variation is more often seen in males but females may also exhibit ARTs. Although multiple phenotypes may exist, male phenotypes are usually categorized into two morphs: a conventional male morph, that expresses secondary sexual characteristics which are preferred by females, or morphs that monopolize access to female; then there is an alternate male morph that does not express the conventional phenotype and shows parasitic behaviors such as sneaking or coercive copulations (Knapp 2004). Examples of ARTs include calling vs. satellite behavior in anurans (Perrill et al. 1978, Fellers 1979, Forester and Lykens 1986, Arak 1988), courting vs. coercive behavior in Poeciliidae (Zimmerer 1982, Travis and Woodward 1989, Kodric-Brown 1993), territorial vs. non-territorial (sneakers, nomads) reptiles (Thompson

and Moore 1991, Sinervo and Lively 1996), mate-guarding vs. sneaker beetles (Emlen 1997a). In addition to the vast phenotypic differences among conspecific males, there is also variation of ARTs among different taxa. However, ARTs can be generally categorized whether phenotypes are fixed or plastic.

ARTs can be categorized according to the genetic basis of expression where ARTs are genetically-mediated polymorphisms, some ARTs are conditionally expressed mediated by an external environmental cue, and other ARTs are mixed strategies (Gross 1996). Another way to categorize ARTs is the degree of flexibility to switch between tactics whether they are genetically or conditionally expressed (Fig. 1; Oliveira et al. 2008b). In this classification scheme, ARTs are either fixed, so that individuals are one tactic throughout their lifetimes and cannot switch tactics, or ARTs show plasticity. Plasticity in ARTs are further divided into simultaneous ARTs, so that individuals can express both tactics at any time, or sequential ARTs, where individuals can express one tactic at a time but in a certain sequence. Sequential ARTs are divided into either fixed sequence where individuals express one tactic when younger and another tactic when older, or a reversible sequence, where either tactic is expressed according to some environmental cue and individuals switch between tactics throughout their lifetimes.

Many studies use body size as an indicator of ARTs and divide male morphs into size classes. Body size is often significantly different between morphs, and oftentimes, behavioral differences between morphs are size-dependent where larger males show conventional morph behaviors by exhibiting sexually-selected traits or by monopolizing females and smaller males show parasitic behaviors (Taborsky 1994). In an obligate shell-brooding cichlid, *Lamprologus callipterus*, the dwarf morph is only 2.5% the size of

the conventional morph (Sato et al. 2004). Fish that stop growing at maturity also show large differences in size, such as live bearing fish in the family Poeciliidae (Ryan et al. 1990, Ryan et al. 1992, Erbelding-Denk et al. 1994). However, body size is also influenced by various other factors including nutrition and stress that are unrelated to mating and often show a continuous distribution. For example, in the horned beetle genus, *Onthophagus spp*., males show a continuous distribution in body size but are dimorphic for horn length. Therefore, quantitative traits such as body size is an important trait that varies with mating tactics and can further contribute to the understanding of discrete variation seen in ARTs.

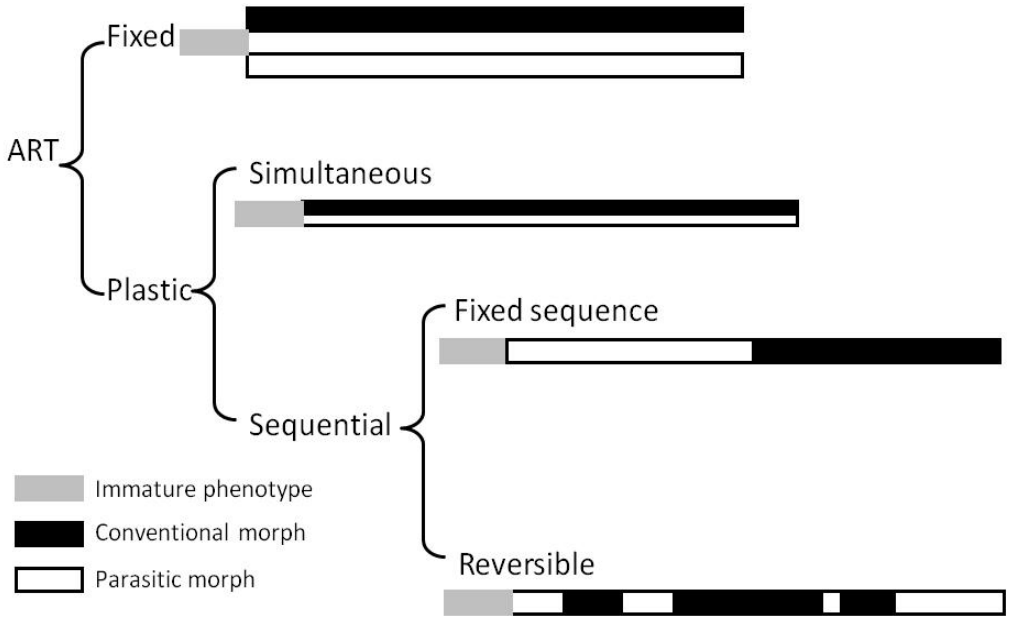

Figure 1.1
ARTs categorized by degree of flexibility (redrawn from Oliveira et al. 2008).

Alternative reproductive tactics has served as models for understanding both genetic and environmental sources of phenotypic variation and how they are mediated by

endocrine mechanisms. In horned beetles, *Onthophagus* spp.*,* expression of large-horned mate-guarding males and small-horned sneaker males are dependent on the quality of nutrition received during juvenile development (Emlen 1997b). Manipulation of larval diets during the critical stage of development is correlated to changes in juvenile hormone, which mediates expression of male horn length (Emlen and Allen 2004). Further studies on populations that differed in horn development thresholds showed that genetic differences between populations were due to divergence in sensitivity to juvenile hormone and timing of that sensitivity (Moczek and Nijhout 2002). Hormones mediate changes in conditionally-expressed ARTS, such as the horned beetles but can also mediate genetically-based ARTs. In side-blotched lizards, *Uta stansburiana*, throat color of three male morphs is a heritable trait expressed by a three allele-one locus system (Sinervo and Zamudio 2001). Orange-throated morphs are aggressive and territorial, while blue-throated males show mate-guarding behavior, and yellow-throated males are sneakers (Sinervo and Lively 1996). Orange-throated males show the highest level of testosterone (T) and experimental increases in T of blue- and yellow- throated morphs elicited orange-throat-like behavior (Sinervo et al. 2000). Hormones are often the critical link that translates the effects of the environment on the genome.

Social control of ARTs

Many systems with ARTs show some degree of social influence on tactic expression, particularly the presence of other males. For example, small-horned sneaker beetles will show mate-guarding behavior in the absence of other males (Emlen 1997b) and experimental removal of the most attractive males in scorpionflies, *Panorpa* spp.,

will induce tactic change in remaining males (Thornhill 1981). In guppies, *Poecilia reticulata*, males are more likely to coerce matings rather than court with females when placed in a male-biased environment (Řežucha and Reichard 2014). Social influences of rival males on ARTs are most apparent in fish that express nuptial coloration during the breeding season. Males exhibit drab coloration when in subordinate or sneaking morph status, and when colorful, dominant males are removed, males often opportunistically change coloration and take-over vacated territories. (e.g., sand goby (Pomatoschistus minutus): Takegaki et al. 2012, African cichlid (Astatotilapia burtoni): Maruska and Fernald 2013, black-faced blenny (Tripterygion delaisi): Schunter et al. 2014). In addition to the presence of other males, the size of potential competitors also affects the expression of ARTs (Engqvist and Taborsky 2016).

Maternal investment into offspring is one form of social influence that may play an important role in expression of ARTs. Mothers may differentially allocate resources to offspring if they can make accurate predictions about their offspring's future competitive environment. Buzatto et al. (2012) found that female dung beetles, *Onthophagus taurus*, reared with conspecifics had conventional "major" morph sons with longer horns but the sons had greater variation in body size than "major" morph sons produced by females reared in isolation. The authors suggested that mothers who experience high population densities would allocate more resources into "major" sons with longer horns. Mothers exposed to stressors may influence expression of ARTs through hormone manipulation to offspring, particularly steroid hormones. In a review by Groothuis & Schwabl (2008), concentrations of hormones in the egg and yolk of avian embryos may be independent of the mother's plasma hormone levels, suggesting maternal manipulation of offspring

hormone exposure. For example, mothers of zebra finch, *Taeniopygia guttata*, increase testosterone in eggs after mating with an attractive male (Gil et al. 1999). Therefore, hormone-mediated maternal effects based on social cues are another avenue that contributes to variation in male ARTs.

Many proximate mechanisms in response to social cues may be associated with expression of ART's. One method to examine the mechanistic basis for tactic expression is through transcriptomics, which is used to study patterns of gene expression profiles in the brain between individuals with alternative phenotypes (Aubin-Horth et al. 2005, Fraser et al. 2014, Schunter et al. 2014, Bohne 2018). More compelling is that differences between the brain transcriptomes of alternative male phenotypes can be greater than the differences between the transcriptomes of males and females overall (Schunter et al. 2014). One study reveals that genes associated with learning and memory are upregulated in sneaker male morphs (Fraser et al. 2014). The authors propose that sneaker males require more cognitive functions to perform sneaker-like behavior such as navigating complex social interactions. Sneaker males may require a greater degree of social competence such as recognizing other individual males and the social status of these males, keep track of receptive females, and be aware of the location of these individuals (Taborsky and Oliveira 2012).

Genetic control of ARTs

Some ARTs show a simple Mendelian mode of inheritance with a one locus system (e.g., Lank et al. 1995, Shuster and Sassaman 1997, Sinervo and Zamudio 2001, Ocana et al. 2014). Other studies show evidence that ARTs may have Y-linked basis of

expression, for example in many teleost fish (Ryan et al. 1992, Erbelding-Denk et al. 1994, Ptacek 2002, Ocana et al. 2014). In male *Xiphophorus*, age to sexual maturity determines male size and is regulated by the P-locus ("pituitary or puberty" locus), located on the sex chromosomes (Kallman and Borkoski 1978, Zimmerer and Kallman 1989). Control of body size in *Xiphophorus* is an exemplary model of how one gene of large effect can result in extreme phenotypic variation. However, early studies on the genetic basis of ARTs were incomplete because of technological limitations. Oftentimes, male ARTs involve a suite of traits constituting a complex phenotype, where the genetic architecture is not simply determined by one locus and scanning the entire genome for candidate loci was not possible. However, next-generation sequencing provides the technology to find regions of the genome that may correlate with phenotypes. Performing a genome-wide association study (GWAS) to understand the genetic architecture of complex traits will enhance our knowledge of the phenotypic variation seen in ARTs (Robinson et al. 2014).

Genome-wide association studies links genetic variation to phenotypic variation by testing the association of a marker(s), typically single nucleotide polymorphisms (SNPs) to a phenotypic trait. A genome-wide association study (GWAS) conducted on ARTs in male Atlantic salmon (*Salmo salar*), showed that several genomic regions varied with phenotypes (Johnston et al. 2014). These associated regions were known to contribute to various aspects of physiology, behavior, and morphology such as metabolism, muscle development, immune response, and mate choice. Quantitative traits such as body size, which is particularly important in the variation among different ART phenotypes, appear to have complex genomic architectures (Gutierrez et al. 2015). Using

genomic association studies in fish, researchers identified genomic regions responsible for body size and growth (Tsai et al. 2015, Yoshida et al. 2017, Yu et al. 2018) as well as identifying previously unknown genes that regulate growth (Li et al. 2018).

One challenging aspect of GWAS is the statistical analysis of the large quantity of genetic variants, such as SNPs, that may be associated with phenotypes of interest (Guan and Stephens 2011). In the simplest form of analysis, a single regression can be used on each SNP to determine whether or not a particular SNP correlates with a trait. However, performing multiple comparisons when there are tens to hundreds of thousands of variant sites, reduces the explanatory power of each comparison by increasing the likelihood of a Type I error (false positive). These studies typically correct for multiple comparisons using methods such as a Bonferroni correction, or estimation of the false discovery rate (FDR). More recently, researchers have implemented multi-marker approaches to analyzing GWAS data (Zhou et al. 2013, Lindtke et al. 2017, Lucas et al. 2018). However, although more powerful than single-SNP analysis, multi-marker analyses pose another set of challenges to researchers. When performing multi-marker regression on GWAS data, there are significantly more predictor variables (i.e. variant sites) than there are samples, referred to as a p>>n problem (West 2003). Analyzing all possible combinations among any possible number of variants is logistically and computationally prohibitive. Additionally, association studies typically face what is known as the missing heritability problem (Maher 2008, Eichler et al. 2010). Finding significant SNPs in association studies should collectively attribute the same percentage of genetic variance that explains phenotypic variance as other heritability estimates such as those derived from family studies. However, heritability estimates for any given trait using family

studies or other methods are typically greater than heritability estimates in GWAS studies. The "missing heritability" is often unaccounted (Yang et al. 2010). Therefore, any approach used to analyze a GWAS data set must address these issues.

Hormonal control of ARTs

Most studies on the endocrine control of ARTs have focused on hormones of the HPG (hypothalamus-pituitary-gonadal) and HPA (hypothalamus-pituitary-adrenal) axes (Fig. 2; Knapp 2004, Oliveira et al. 2008b). Sex steroids such as androgens, and stress hormones such as glucocorticoids (GCs) have emerged as primary candidate hormones in the study ARTs (reviewed in Oliveira et al. 2008a). Androgens affect male morphology and reproductive behavior (Oliveira 2004, Hirschenhauser and Oliveira 2006, Leary 2009) while GCs, such as cortisol, can mediate social interactions in a reproductive context (Husak and Moore 2008, Schreck 2010). Both androgens and GCs share some regulatory mechanisms and may work in concert to mediate ARTs (Leary and Knapp 2014). Androgen and GC synthesis show competitive inhibition of the enzyme 11βHSD (11-hydroxysteroid dehydrogenase), which regulates expression of reproductive traits and behaviors (Sapolsky et al. 2000, Knapp 2004, Pradhan et al. 2014). Additionally, steroid binding globulins (SBGs), which bind to circulating steroids and regulate the availability of these steroids to target tissues, may play a role in the interaction between the HPG- and HPA-axes (Knapp 2004, Oliveira et al. 2008a). One study found the presence of a SBG, named androgen-glucocorticoid-steroid-binding globulin (AGBG) that has binding affinity to both sex steroids (testosterone and progesterone) and corticosterone in tree lizards, *Urosaurus ornatus*, (Jennings et al. 2000).

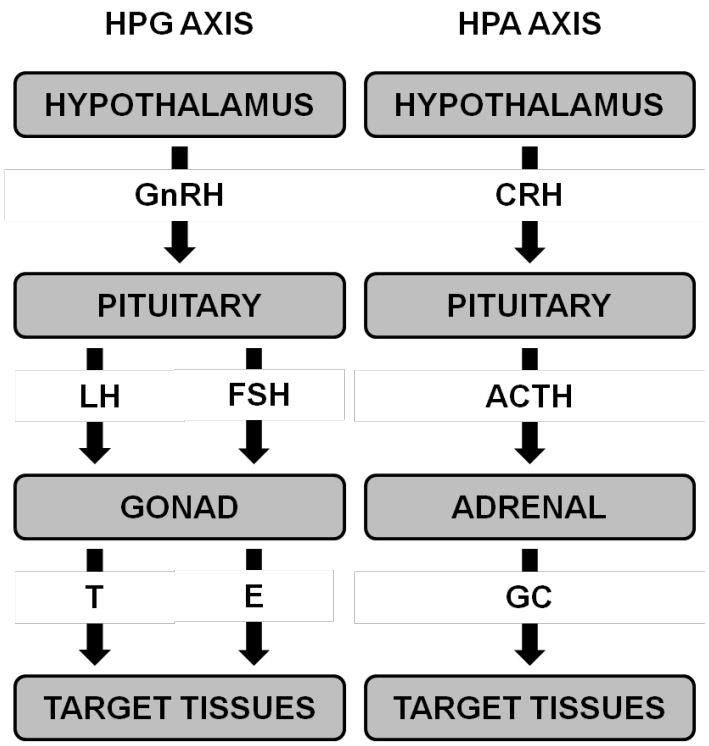

Figure 1.2
Hormone pathways for reproduction (HPG axis) and stress (HPA axis). Abbreviations for hormones of the HPG axis: GnRH (gonadotropin-releasing hormone), LH (luteinizing hormone), FSH (follicle-stimulating hormone), T (testosterone), E (estrogen). Abbreviations for hormones of the HPA axis: CRH (corticotropin-releasing hormone), ACTH (adrenocorticotropic hormone), GC (glucocorticoid).

Androgens such as testosterone or 11-ketotestosterone (KT; the primary androgen of teleost fish), are known to mediate male reproductive functions and behavior (Oliveira 2004). In species with ARTs, the conventional morph is predicted to have greater levels of androgens than the alternative morph, which is supported by several studies (Sinervo et al. 2000, Fagundes et al. 2012, Leary and Harris 2013). However, not all species show this pattern - some species show no difference in androgen levels between morphs (Leary et al. 2004, Baird and Hews 2007, Smith et al. 2015) while other species show that the alternative morph has greater levels of androgens (Mendoça 1986, Duckworth et al. 2004). The direction of androgen differences between morph may be due to the specific

nature of the ARTs. For instance, in some ARTs, the alternative morphs have bigger gonads than the conventional morph, as predicted by sperm competition hypothesis (Taborsky 1998). In these cases, higher levels of androgens may be found in alternative morphs because androgens are known to stimulate spermatogenesis (Schulz and Miura 2002). Conversely, higher levels of androgens can be found in conventional morphs in ARTs where conventional morphs have greater aggression to defend territories and face greater social challenges (Hirschenhauser and Oliveira 2006). Subsequently, ARTs that have both types of morphs (i.e., a conventional morph with greater social challenges and an alternative morph with bigger gonads) may see no difference in androgens.

Social and environmental cues may change circulating levels of GCs, thereby mediating expression of plastic ARTs (Leary and Knapp 2014). Glucocorticoids have well-known suppressive effects on reproduction and behavior by decreasing GnRH release from the hypothalamus (Dubey and Plant 1985) and decreasing concentration of LH receptors in the gonads thereby reducing gonadal sensitivity to LH (Bambino and Hsueh 1981). These physiological changes can then result in decreased sexual receptivity. However, GCs may also mobilize energy stores to stimulate metabolically demanding aspects of reproductive behaviors, such as courtship displays or facing challenges by other males (Emerson and Hess 2001, Leary and Knapp 2014, Reedy et al. 2014). According to Creel (2005), the morph that faces more social challenges is predicted to have greater levels of GCs, which is typically the conventional morph. These males typically fight other males for territories and monopolize mates.

When faced with other reproductive rivals, males can show elevated GCs due to a potential aggressive interaction associated with male-male competition. For example, in

convict cichlids, *Amatitlania nigrofasciata*, males showed a correlation in GCs and intensity of fights (Earley et al. 2006). The authors also found higher pre-fight levels of GCs compared to post-fight levels that may indicate acute stress from the anticipation of an aggressive interaction. Alternatively, males show increases in GCs due to a greater intensity of energetic courtship displays (Emerson and Hess 2001, Leary and Harris 2013). Differences in GC levels among individuals can then influence behavioral decisions such as engaging in aggressive interactions for social dominance (Creel 2001). In olive baboons, *Papio anubis*, dominant males had lower basal levels of GCs but that during times of social instability within their social groups, all males had higher levels of GCs (Sapolsky 1983). Furthermore, Earley & Hsu (2008) found that in the mangrove killifish, *Kryptolebias marmoratus*, individuals with lower GCs initiated contests, delivered more attacks, and had more wins than individuals with higher GCs. Therefore, the presence of rival males may alter GC levels of males and subsequently affect reproductive behaviors. The morph that is predicted to experience greater social challenges should also show greater levels of GCs.

One proposed mechanism for the suppressive effects of GCs at higher concentrations is through the saturation of different GC receptors on the target tissues. Type I receptors have a higher affinity for GCs than type II receptors (de Kloet et al. 1993). GCs would only be bound to type I receptors at low or baseline levels. When GC levels increase, type I receptors become saturated and binding switches to type II receptors, which then signal suppression of HPG axis. Although suppression of reproductive behavior is a well-established effect of GCs, there appears to be limited evidence for GCs mediating or stimulating reproduction (Sapolsky et al. 2000). The role

of GCs as facilitators or inhibitors of reproductive function is still currently under debate (Mommsen et al. 1999, Milla et al. 2009).

Studies that have investigated both androgens and GCs on ARTs have mixed results. In amphibians, Leary et al. (2004) found that calling males of both the Great Plains toad, *Bufo cognatus*, and Woodhouse's toad, *Bufo woodhousii*, had higher corticosterone (CORT) levels than satellite males but found no difference in androgens between morphs. However, Leary & Harris (2013) found that in green treefrogs, *Hyla cinerea*, calling males had higher androgens and lower CORT than satellite males. Lastly, calling territorial male bullfrogs, *Rana catesbeiana*, had lower androgens and higher CORT than satellite males (Mendoça 1986). In tree lizards, with two male morphs, only satellite orange males have testosterone levels that are sensitive to suppression by CORT, whereas territorial orange-blue males have testosterone levels that are resistant to the effects of CORT (Knapp and Moore 1997). In a recent review, Leary & Knapp (2014) suggest that GCs may mask the effects of androgens on male reproductive phenotype. These studies suggest that the relationship between androgens and GCs are complicated and warrant further investigation.

Study system: *Poecilia latipinna*

The sailfin molly, (*Poecilia latipinna*), is a live-bearing fish species found in freshwater and brackish habitats throughout coastal areas surrounding the Gulf of Mexico and the southern Atlantic coast (Page and Burr 1991). Sailfin mollies show sexual dimorphism, where females show continuous growth and show little to no variation in their gray coloration and mature males exhibit extreme variation in body size, shape,

coloration, and behavior (Snelson 1985, Ptacek and Travis 1996). Male sailfin mollies vary in body size according to the time to sexual maturity, after which males do not continue to grow (Fig. 3; Snelson 1982, Travis et al. 1989). Body size varies greatly but shows a continuous distribution within populations (Travis 1994b, a). Males also have a sail-like dorsal fin that scales to body size in a positive allometric relationship so that larger males have disproportionately larger dorsal fins (Farr et al. 1986). Male gonopodium (intromittent sexual organ) may scale in a negative allometric fashion to body size because sperm competition plays an important role among males of different sizes (Aspbury 2007). Therefore, smaller males likely have disproportionately larger gonopodium relative to their body size. Larger males typically exhibit striking coloration (yellow and black coloration with iridescent blues) and perform courtship behaviors (Travis and Woodward 1989, Ptacek and Travis 1996). Conversely, smaller males do not have exaggerated secondary sexual characteristics or coloration and perform 'sneaking' behavior through forced copulations. Variation in male body size and behavior appears to be a Y-linked trait particularly at the extreme ends of variation (Trexler and Travis 1990, Trexler et al. 1990, Travis 1994a, Ptacek 2002). However, intermediately-sized males show a great degree of plasticity in behavior depending on the relative size of males within the social environment (Travis and Woodward 1989, Fraser et al. 2014). Furthermore, intermediately-sized males showed greater changes in brain expression profiles when in different social contexts (alone or with other males) than small males in the same social contexts (Fraser et al. 2014). Therefore, expression of phenotype and behaviors in male sailfin mollies appears to be mediated by both genetic and environmental factors.

Figure 1.3
Photograph of the extreme ends in male phenotypic variation.

Mutual mate choice exists in the sailfin mollies where female sailfin mollies prefer larger males and males prefer to mate with and associate with larger females (Marler and Ryan 1997, Ptacek and Travis 1997, Gabor 1999, Gabor and Ryan 2001). Females also prefer males with a disproportionately large dorsal fin relative to body size (Ptacek 1998, MacLaren et al. 2004). Although females prefer larger males, which exhibit more courtship behaviors, females do not prefer males that court more per se (Ptacek and Travis 1997). Females also prefer to associate with males under full UV spectrum lighting versus UV-filtered light, suggesting a component of male coloration may play a role in female mate choice (Palmer and Hankison 2014). Male mate choice in sailfin mollies is particularly important because they are sexually parasitized by a unisexual heterospecific species, the Amazon molly, *P. formosa*. The Amazon molly is a gynogenetic species of fish that most likely arose from a sexually reproducing hybrid between male sailfin mollies and female Atlantic mollies, *P. mexicana* (Hubbs and Hubbs

1932, Avise 2008, Alberici da Barbiano et al. 2013). Gynogens are all female lineages that require sperm from males of closely related species to initiate embryogenesis, but inheritance is strictly maternal. Evolutionary persistence of gynogens requires mate recognition errors by males of the bisexual species, but selection should favor males that are able to discriminate against heterospecific matings (Ryan et al. 1996, Gabor et al. 2012). Gabor & Ryan (2001) found male sailfin mollies prefer conspecific females and they also found no correlation between male size and male mate choice. However, the authors did not directly test for the effects of male size on male mate choice. Species recognition by male sailfin mollies may be mediated by changes in 11-ketotestosterone (11-KT). Gabor & Grober (2010) measured 11-KT response (post-mating/pre-mating hormone levels) of both males and females and found that male and female sailfin mollies show a considerable 11-KT response when they mate with each other. However, when male sailfin and Amazon mollies mate with each other, there was no corresponding 11-KT response in either the male sailfin molly or the Amazon molly.

Here I investigate aspects of the social, hormonal, and genetic effects on variation in male phenotype of sailfin mollies. In Chapter 2, I examine the effects of other rival males on male mate choice for conspecific females, and on the changes in release rates of 11-KT and cortisol within a mate choice context. Then in Chapter 3, "Individual variation in ACTH-induced cortisol levels in females of a livebearing fish at different gestational stages", I investigate how cortisol release rates in female sailfin mollies varied across gestational stages. I conduct an ACTH-challenge to induce increases in cortisol release rates to determine the natural range of cortisol by gestating females and how much individual variation within and among females affected cortisol release rates. This study

aims to identify provide insight on potential effects of maternal stress on male offspring. Lastly, in Chapter 4, I study the underlying genomic architecture of male alternative phenotypes using a genome-wide association (GWAS) study with a Bayesian sparse linear mixed model approach. I identify morphological traits that either define alternative male phenotypes or were previously shown to have a genetic basis (Loveless et al. 2010). Together, these chapters will provide greater understanding of the genetic and physiological mechanisms for the variation in male phenotypes of sailfin mollies.

II. RIVAL MALE DOES NOT AFFECT MALE MATE CHOICE OR CORTISOL BUT DOES AFFECT 11-KETOTESTOSTERONE IN A UNISEXUAL-BISEXUAL MATING COMPLEX OF FISH

Male mate discrimination may be affected by the social environment (presence or absence of rival males or mates), which can also affect stress and sex hormones (e.g., cortisol and 11-ketotestosterone (11-KT)). Amazon mollies, Poecilia formosa, is an all-female fish species dependent on sperm from mating with male P. latipinna. We investigated male mate choice in P. latipinna between conspecific females and P. formosa with a rival male present and no rival male present. We measured cortisol and 11-KT release rates from all fish. The presence of a rival male had no effect on male mate choice for conspecific females nor overall mating effort. Male 11-KT decreased on the second day after exposure to a rival male on the first day. Focal male 11-KT is positively correlated with the size of the rival male. Both conspecific and heterospecific females released more 11-KT when in the presence of a rival male than when not. Neither male nor female cortisol was affected by the presence or absence of the rival male. We did not find an effect of rival males on male mate choice in contrast to our prediction. Instead, our findings may indicate a hormonal response to social competition.

Introduction

In some systems, individuals may obtain fitness benefits from heterospecific matings (Pfennig 2007; Schlupp et al. 1994). The social environment can strongly influence individual mating decisions and preferences even when the mating choice seems maladaptive (West-Eberhard 1983). For example, when mate-choice copying occurs, individuals increase preference for conspecific mates that are preferred by other individuals, including heterospecifics (Auld and Godin 2015; Schlupp et al. 1994). In addition, audience effects occur with the presence of a mating rival and can change mating preferences for conspecific or heterospecific partners (Auld and Godin 2015; Mautz and Jennions 2011; Plath et al. 2008; Plath et al. 2008). For example, in Poecilia mexicana, males reduce overall mating activity, decrease preference for conspecific females, and initiate mating with heterospecific females, when in the presence of rival male (Plath et al. 2008). Audience effects are mediated by various physiological processes (Aspbury 2007; Cummings et al. 2008; Desjardins et al. 2015), but little is known about the hormonal basis to changes in mating preferences. Understanding the hormonal mechanisms that mediate these mating and social behaviors can help us elucidate how the social environment affects mating behaviors. Social environments of animals often include competitive interactions which can mediate changes in concentrations of androgenic and glucocorticoid hormones (reviewed by Briffa and Sneddon 2007; Oliveira 2004; Schreck 2010; Teles and Oliveira 2016).

In teleosts, one of the primary androgens, 11-ketotestosterone (11-KT), regulates male mating behavior (Borg 1994), male response to social challenges (Clement et al. 2005; Hirschenhauser et al. 2004), and may mediate species recognition in male mate

choice (Gabor and Grober 2010). Social dominance, male ornamentation or coloration may also correlate with higher 11-KT levels in fish (Butts et al. 2012; Cardwell and Liley 1991; Oliveira et al. 2008). Sex steroid hormone receptors are found in key brain regions known to modulate social behaviors in teleost fish and across vertebrates (Munchrath and Hofmann 2010) indicating a potentially strong role of androgens in the effects of social competition on mate choice. Glucocorticoids, such as cortisol, are involved in the stress response and have more complex effects on reproduction (Milla et al. 2009). Increases in cortisol decreases selectivity in mate choice, reduce sexual receptivity, and suppress sexual behavior of subordinates (Davis and Leary 2015; Vitousek and Romero 2013). However, small increases in cortisol may also allow individuals to mobilize energy stores for metabolically demanding aspects of reproductive behaviors, such as courtship displays or facing challenges by other males (Clement et al. 2005; Teles and Oliveira 2016).

A unique system for investigating hormonal modulation of social interactions and species recognition is a unisexual-bisexual complex of fish, where females of a unisexual species rely on matings with closely related males of a bisexual species. The Amazon molly, Poecilia formosa, is a gynogenetic livebearing species of fish that most likely arose from a hybrid crossing between male P. latipinna and female P. mexicana (Alberici da Barbiano et al. 2013; Avise 2008; Hubbs and Hubbs 1932; Warren et al. 2018). Gynogens are all-female lineages that require sperm from males of closely related species to initiate embryogenesis, but inheritance is strictly maternal. Evolutionary persistence of gynogens requires matings by males of the bisexual species. Both male P. latipinna and P. mexicana prefer to mate with conspecific females over female P. formosa, but this

preference is stronger in male P. latipinna than in male P. mexicana (Gabor et al. 2012; Gabor and Ryan 2001; Ryan et al. 1996). Mating systems differ between these two closely-related species: male P. latipinna exhibit alternative mating tactics, whereas male P. mexicana show a dominance hierarchy (Farr et al. 1989; Ptacek 1998). Male P. latipinna have extreme continuous variation in a suite of morphological and behavioral traits (Snelson 1985). At one end of the variation, large males typically exhibit striking coloration, an exaggerated sail-like dorsal fin, and perform courtship behavior (Ptacek and Travis 1996; Travis and Woodward 1989). Female P. latipinna prefer to mate with large males (MacLaren et al. 2004; Ptacek 1998). Conversely, smaller males do not have exaggerated secondary sexual characteristics and are more likely to secure matings via coercive (e.g., forced copulation) behavior. However, intermediate-sized males exhibit a great degree of plasticity in behavior depending on the relative size of males within the social environment (Fraser et al. 2014; Travis and Woodward 1989).

The presence of a rival male influences male reproductive behavior in P. mexicana and both reproductive behavior and physiology in P. latipinna. In P. mexicana, presence of a rival conspecific male significantly decreases a male's initial mate preference, but males retain their initial choice when there is no rival male present (Plath et al. 2008). Male P. latipinna prime more sperm prior to mating and expend more sperm when mating with conspecific females in the presence of male competitors, suggesting that males respond physiologically to sperm competition risk (Aspbury 2007). Furthermore, Gabor and Grober (2010) measured male and female P. latipinna 11-KT-response (post-mating/pre-mating hormone release rates) and found that both sexes show an increase in 11-KT-response when they mate with each other but this response is absent

when male P. latipinna mate with the unisexual P. formosa. Populations of P. latipinna form loose social aggregations called shoals, which provide ample opportunities for audience effects, mate-choice copying, and other social behaviors (Schlupp and Ryan 1996).

Sperm competition risk theory and empirical findings (e.g., Aspbury 2007), as well as audience effects (Plath et al. 2008a; Plath et al. 2008b), suggest that the presence of a rival male can affect male mating behavior and physiology. Here we test the hypotheses that the presence of a rival male affects: (1) male mating effort and male conspecific mate choice, and (2) androgen (11-KT) and glucocorticoid (cortisol) responses of male and female P. latipinna. We predict that, in the presence of a rival male, male P. latipinna will show a higher overall mating effort and increase mating attempts with heterospecific females. Additionally, we predict that the presence of a rival will increase cortisol production of male P. latipinna, but not females, as a function of the social challenge. Finally, we predict that, in the presence of a rival male, male P. latipinna will have more 11-KT than males not in the presence of a rival male. Any increases in 11-KT of males with rivals may also lead to increases in 11-KT production of conspecific females that are paired with males in the presence of rivals as was shown in a previous study (Gabor and Grober 2010).

Materials and methods

Animal collection and maintenance

We collected P. latipinna and P. formosa from a sympatric population in Northern Tamaulipas, Mexico (25.11°N, 97.56°W) in September 2012 and brought them back to

laboratory facilities at Texas State University, San Marcos, TX. We quarantined fish for 90 days and maintained fish in 37.8 L aquaria (54 x 29 x 33 cm) at a constant temperature (25°C) on a 14:10h light-dark cycle with UV fluorescent lighting. We fed fish twice daily with fish food (Purina AquaMax 200) and supplemented with live brine shrimp nauplii. Prior to testing, we isolated females of both species from males for a minimum of 30 days to standardize levels of receptivity in females. We isolated males for 7 days prior to testing. We performed behavioral experiments from 0700-1500 h, June - August 2013. We only used mature females (≥ 32 mm in standard length; SL) across all trials (Robinson et al. 2011). All research with animals was conducted with approval from Texas State University Institutional Animal Care and Use Committee (IACUC) under protocol #0815_0319_19.

Experimental design

We tested male mate choice for conspecific or heterospecific females in two treatments using a repeated measures design: with a rival and without a rival male. We tested males in both treatments across two days of testing and randomized the order that focal males received each treatment. We divided a 37.8 L test tank into three separate, unequal-sized compartments (Fig. 1). We placed individual focal males (n = 25) in the test tank with a filter, separated with a clear divider from size-matched conspecific and heterospecific females for 21 hours. Conspecific and heterospecific females did not differ in SL (mean ± S.E. = P. formosa: 35.63 ± 0.46; P, latipinna: 35.75 ± 0.45; Wilcoxon Signed Rank Test: V = 171.5, p = 0.315). We placed a rival male or no male, depending on the treatment, at the back third section of the tank separated by a clear divider. The

focal males and rival males did differ in SL (mean ± S.E. = Focal males: 36.52 ± 0.72; Rival males: 27.75 ± 0.45; Wilcoxon Signed Rank Test: $V = 300$, $p < 0.001$). The divider was perforated to allow for both visual and chemical cues to be transferred between all fish.

The following day, we collected water-borne hormones by placing the focal male and both conspecific and heterospecific females in individual 250 mL sterile beakers with 100 mL of de-chlorinated water for 1 hour to measure hormone release rates (following methods of Gabor and Grober 2010). Water-borne hormone collection is a non-invasive method to obtain hormone release rates using repeated measures without compromising health and behaviour. We then returned the focal male and both females to the testing tank, removed the filter, and removed the divider to allow these fish to freely interact. During rival treatment mating trials, the rival males were left in their separate compartments. We recorded focal male mating attempts (gonopodial thrusts) toward conspecific and heterospecific females for 25 minutes. After the mating trial, we returned the filter to the tank and restored the divider to separate the focal male from a new pair of conspecific and heterospecific females. After 21 hours, we repeated the hormone collection and mating trial as described above with the other treatment (rival male present or rival male absent, Fig. 2). Thus, each male ($n = 25$) was tested twice in random order. We did not use focal males and rival males that were housed together in the same trial. We stored all water-borne hormone samples at -20°C until hormones could be assayed (Ellis et al. 2004).

Hormone extraction and assay

We extracted hormones using a solid-phase extraction (SPE) protocol and assayed using enzyme immunoassay (EIA) methods (modified from Gabor and Grober 2010). The correlation between water-borne hormone release rates and plasma steroid levels were previously validated for both cortisol and KT in P. latipinna (Gabor and Contreras 2012; Gabor and Grober 2010). Briefly, we extracted hormones from water samples using Sep-Pak C18 columns (Waters Corp., Milford, MA) placed on a vacuum manifold. We activated columns with 4 mL washes of methanol, followed by 4 mL washes of distilled water. We then ran our water-borne hormone sample through the C18 column to collect hormones and eluted hormones using 4 mL of methanol from C18 columns into borosilicate test tubes. We evaporated the eluent using nitrogen gas and resuspended the hormone residue with 5% ethanol and vortexed then added 95% EIA buffer. We assayed hormones using EIA kits (Cayman Chemical, Ann Arbor, MI) for cortisol and 11-KT. We adhered to protocols provided by the manufacturer for duplicate samples on 96-well plates, which we read on a spectrophotometer at 412 nm (Powerwave XS, Bio Tek Instruments, Inc., Winooski, VT). We ran 11 plates which included a control sample (a pooled mix of hormone suspension from many P. latipinna) across all plates and determined 12.5% inter-assay variation with a range of 0.5% to 15.8% for intra-assay variation for 11-KT. The inter-assay variation for 9 cortisol EIA plates was 14.6%, and the intra-assay variation ranged from 3.6% to 18.5%. Plate sensitivity for minimum 11-KT was 1.3 pg/mL and 35 pg/mL for cortisol.

Statistical analyses

We standardized hormone release rates to SL (standard length) for each fish by

multiplying the hormone release rates (pg/mL) by the reconstitution volume of the hormone residue (1 mL), dividing by SL (mm), and then ln-transformed the data to better fit the assumptions of parametric analyses (see Table 1 for non-corrected hormone values). We conducted all analyses in R version 3.2.3 (R Core Development Team, 2015). We first tested whether there was male mate preference for conspecific females using a paired Wilcoxon Signed Rank test with gonopodial thrusts as the response variable and female species as the predictor variable. As has been found for many sympatric populations (Gabor and Grober 2010; Gabor and Ryan 2001), male P. latipinna mated more often with conspecific than with heterospecific females (mean thrusts ± S.E. = conspecific: 14.54 ± 3.55; heterospecific: 0.88 ± 0.25, V = 139, p = 0.003).

To determine the effects of a rival on male mate choice, we used a generalized linear mixed model with the glmmPQL function from the MASS package (Venables and Ripley 2002) with the number of mating attempts (gonopodial thrusts) directed at females as the response variable. We used a quasi-Poisson distribution because our initial analysis with a Poisson distribution for count data (number of gonopodial thrusts) revealed that the data were overdispersed. We included the following fixed effects: species of female, rival treatment, treatment order, and all interactions. Male identity was included as a random factor.

We also tested the hypothesis that treatment (rival male presence or absence) affects male hormone release rates. We used two linear mixed effect models with the lme function from the nlme package (Pinheiro et al. 2018) with male hormone release rates (11-KT and cortisol) as the response variables. We used rival treatment, treatment order, and their interaction as fixed effects, and male identity as a random factor. Female

species was not included as a factor, as we only had a measure of male hormones when the male was in the presence of both species of female simultaneously. We used a simple regression to determine the relationship between male 11-KT release rates and SL of the rival male.

Similar to above, we also tested the hypothesis that treatment (rival male presence or absence) can affect female hormone release rates. We used two linear mixed effect models with female hormone release rates (11-KT and cortisol) as the response variables. We used species of female, rival treatment, treatment order, and all interactions as fixed effects, and male identity as a random factor to account for both non-independent observations of female species, and for repeated measures between treatments.

Results

There were no significant model effects or interactions on male P. latipinna mating attempts to female P. latipinna or P. formosa (Table 2). The presence of a rival male did not affect overall mating effort of the focal male to either of the females (main effect of Rival Treatment in GLMM, Table 2).

Males that did not encounter a rival on the first day had significantly higher 11-KT than males without a rival on the second day (treatment x order effect: Table 3, Fig. 3). Post-hoc comparisons showed significant decreases in male 11-KT release rates on the second day regardless of treatment order (Fig. 3). There was also a significant positive relationship between the size of the rival male and 11-KT release rates by the focal male ($R2 = 0.34$, $p = 0.002$, Fig. 4). The presence of a rival, treatment order, or their interaction did not affect male cortisol release rates (Table 3), and there was also no

significant relationship between rival male size and cortisol release rates ($R2 = 0.03$, $p = 0.420$).

Female 11-KT release rates were higher in the presence of a rival male (and did not differ between the two species), but were not affected by any of the other model predictors or interactions (Table 4, Fig. 5). Female cortisol release rates were not affected by the presence of a rival, treatment order, species of female, or any of the interactions (Table 4).

Discussion

Understanding the proximate basis of audience effects will further elucidate how the social environment affects mating behaviors. Similar to other studies (Gabor et al. 2013; Gabor and Ryan 2001), we show significant male preference for conspecific females based on male mating attempts. Male P. latipinna mating preference for conspecific females seems to be ubiquitous across P. latipinna populations. However, we did not find support for our hypothesis that the presence of a rival male would affect male mating preference for conspecific over heterospecific females. Although mate-choice copying among females exists in this species (Schlupp et al. 1994), males do not mislead their potential competitors as seen in P. mexicana, where males show reduced preference for conspecific females in the presence of a rival male (Plath et al. 2008; Plath et al. 2008). Male P. latipinna may not have a reduced conspecific mate preference with a rival male because they have a stronger overall conspecific mating preference than P. mexicana (Ryan et al. 1996). If male P. latipinna have a strong conspecific mate preference, then a slight decrease of this initial preference may not be detectable (i.e., a

decrease in a strong preference results in a weaker preference, but still results in an overall preference for conspecific females). However, a decrease in a weak conspecific preference would possibly lead to the expression of either no preference, or a switch to a heterospecific preference as found with P. mexicana. In addition, the presence of a rival male did not affect the overall mating effort of males. Focal males in our study were exposed to a rival male for 17-21 hours prior to the mating trials, which may have been enough time for them to behaviourally habituate to the presence of rival males thus, unintentionally, diminishing their response to rivals.

We also predicted that the presence of a rival male would elicit an increase in 11-KT release rates. Indeed, we found that the presence of a rival male affected 11-KT release rates, but the relationship between a rival male's presence and 11-KT was time-dependent. In the no rival treatment, males had greater 11-KT release rates on day one than males with no rival on day two. Prior studies have shown that isolated males have lower or no difference in androgens levels than males faced with a rival (Dijkstra et al. 2011; Galhardo and Oliveira 2014), which is counter to our results of the higher 11-KT release rates in the no rival male treatment on the first day as compared to the second day. In our study, male 11-KT decreased on day two of the experiment regardless of treatment possibly due to down-regulation of 11-KT. However, there was greater down-regulation in the no-rival male treatment than the rival treatment, suggesting a relationship between the presence of a competitor and 11-KT.

After 34-42 hours (day two of the experiment), we observed a reduction in overall male P. latipinna 11-KT release rates. One hypothesis is that male 11-KT is down-regulated after initial increases from exposure to a new social environment. Data on the

timing of 11-KT changes in response to social challenges or to the presence of mates in fish species are not universally consistent. Males of several cichlid species show increases in 11-KT after one hour of exposure to a simulated territorial intruder (Hirschenhauser et al. 2004), and shoaling male zebrafish have increased 11-KT release rates 30 minutes after males engage with rival males (Teles and Oliveira 2016). However, there is no difference in 11-KT of nest-holding male Siamese fighting fish, Betta splendens, 20 minutes after treatment with or without a male audience, but 11-KT is significantly lower in the presence of a female audience (Dzieweczynski et al. 2006). These studies suggest that changes in 11-KT can occur at relatively shorter time scales in response to the presence of social rivals or mates, but our study suggests that overall release rates of 11-KT decrease after longer time periods, which can mask any effects of social rivals on male release rates of androgens.

Focal male 11-KT release rates are positively correlated with the size of the rival male. Male P. latipinna have alternative mating phenotypes, they vary greatly in body size and they also engage in aggressive interactions that include chasing, nipping, and aggressive displays. Larger males are preferred by females (Ptacek and Travis 1997) and may pose a greater threat in mating competition which could explain increases in 11-KT of focal males in the presence of larger rival males. Audience effects on male mate choice are greater when males are confronted with large rivals (Auld et al. 2017; Bierbach et al. 2011). In the shell-brooding cichlid, Lamprologus callipterus, large nest-holding males increased 11-KT when confronted with other large nest holders or intermediately sized sneakers, but not when confronted with the much smaller dwarf male (von Kuerthy et al. 2016). The relative size or competitive ability of rival males may have an important role

in the androgen response of males, which could be explored in future studies.

Female P. latipinna and P. formosa, in our study, show an increase in 11-KT in the presence of a rival male. This increase in female 11-KT is relatively smaller than the changes seen in the focal male 11-KT. Androgens are predominantly associated with male physiology and behavior but increases in female 11-KT release rates may be a physiological byproduct in response to mating interactions (Stacey 2003, 2015) or may allow males to discriminate between species (Gabor and Grober 2010). Although we found small increases in female 11-KT release rates, we did not find any differences in 11-KT release rates between the two species of female. We interpret this result with caution because our result does not match the results of (Gabor and Grober 2010), who found increases in 11-KT of conspecific females when mated with male P. latipinna, but no such increase in P. formosa that mated with male P. latipinna. In the prior study, Gabor and Grober (2010) tested males with one species of female at a time (i.e., sequential mate choice trials) which may explain differences between our results. The presence of both species of females in our study (i.e. simultaneous mate choice trials) may further affect female hormones and suggests that males would have greater difficulty in using 11-KT release rates of females as a cue for species identification in natural populations.

We found no support for the hypothesis that male and female (both conspecific and heterospecific) cortisol release rates are affected by the presence of a rival male. Cortisol plays a role in short-term mobilization of energy stores for energetically demanding mating behaviours, such as courtship and male-male aggression (Wingfield and Sapolsky 2003). One possible reason for a lack of differences between the rival

present and the rival absent treatments in cortisol release rates in our focal fishes is because the rival male was never in direct physical contact with them. Male zebrafish, Danio rerio, do not have higher cortisol release rates when faced with mirrors and male chemical cues, but do have higher cortisol release rates when they are allowed to directly compete with rival males and win in social competitions (Teles and Oliveira 2016). Another hypothesis for the lack of variation in cortisol across the rival male treatments is because of high male 11-KT release rates, especially on the first day of testing. In trout, 11ß- hydroxysteroid dehydrogenase (11ß-HSD) catalyzes 11-KT production but may also play a role in protecting the gonad tissue from circulating cortisol (Fernandino et al. 2013).

Although the social environment is an important component of male mating behavior in other species, we found no evidence to support the hypothesis that the presence of a rival male affects species recognition in mate choice of male P. latipinna. However, we did find that the social environment has an effect on male physiology. The presence of a single rival male is enough to elicit a change in male androgen release rates, which may translate into changes in behaviour in subsequent encounters with other rival males or females. In addition, males may not be able to discriminate between species when in a complex social environment such as the set-up in this study where both species of females are presented together, possibly due to both females releasing similar amounts of 11-KT. This result could partially account for the maintenance of the unisexual species in this system.

Tables and table legends

Table 2.1
Mean ± S.E. hormone release rates (pg/mL) of males and female fish.

	Mean	S.E.
Male 11-KT (pg/mL)	313.87	82.89
Male cortisol (pg/mL)	16593.26	1440.44
Female *P. latipinna* 11-KT (pg/mL)	2.24	0.34
Female *P. formosa* 11-KT (pg/mL)	2.00	0.17
Female *P. latipinna* cortisol (pg/mL)	9136.46	1384.00
Female *P. formosa* cortisol (pg/mL)	11310.72	1768.70

Table 2.2
Fixed effects from a quasi-Poisson GLMM examining social effects on male *P. latipinna* mating attempts (gonopodial thrusts) with male identity as a random factor.

x	Estimate ± S.E.	t	*p*
Female Species	-3.404±1.92	-1.769	0.081
Rival Treatment	-0.080±1.65	-0.049	0.961
Treatment Order	-0.812±0.61	-1.335	0.186
Female Species x Rival Treatment	2.009±2.59	0.775	0.441
Female Species x Treatment Order	0.475±1.27	0.375	0.709
Rival Treatment x Treatment Order	0.381±1.11	0.344	0.732
Female Species x Rival Treatment x Treatment Order	-1.635±1.91	-0.858	0.394

Table 2.3
Fixed effects from a linear mixed effects model examining social effects on male *P. latipinna* hormone release rates with male identity as a random factor. Significant *p*-values are in bold.

Male	Estimate ± S.E.	t	*p*
11-KT			
Rival Treatment	2.672±1.26	2.128	**0.037**
Treatment Order	0.282±0.43	0.652	0.516
Rival Treatment x Treatment Order	-1.855±0.83	-2.226	**0.029**
Cortisol			
Rival Treatment	0.313±1.00	0.313	0.755
Treatment Order	-0.497±0.36	-1.382	0.171
Rival Treatment x Treatment Order	-0.022±0.66	-0.033	0.974

Table 2.4
Fixed effects from linear mixed models examining social effects on female hormone release rates with male identity as a random factor. Significant *p*-values are in bold.

Female	Estimate ± S.E.	t	*p*
11-KT			
Female Species	0.825±0.55	1.492	0.141
Rival Treatment	1.392±0.68	2.037	**0.046**
Treatment Order	0.188±0.28	0.674	0.503
Female Species x Rival Treatment	-1.299±0.76	-1.702	0.094
Female Species x Treatment Order	-0.274±0.34	-0.803	0.425
Rival Treatment x Treatment Order	-0.769±0.44	-1.743	0.086
Female Species x Rival Treatment x Treatment Order	0.487±0.48	1.010	0.316
Cortisol			
Female Species	0.299±0.83	0.362	0.719
Rival Treatment	1.138±1.00	1.141	0.259
Treatment Order	0.168±0.41	0.409	0.684
Female Species x Rival Treatment	0.043±1.15	0.037	0.971
Female Species x Treatment Order	-0.082±0.51	-0.160	0.874
Rival Treatment x Treatment Order	-0.803±0.64	-1.250	0.217
Female Species x Rival Treatment x Treatment Order	0.042±0.72	0.059	0.954

Figures and figure legends

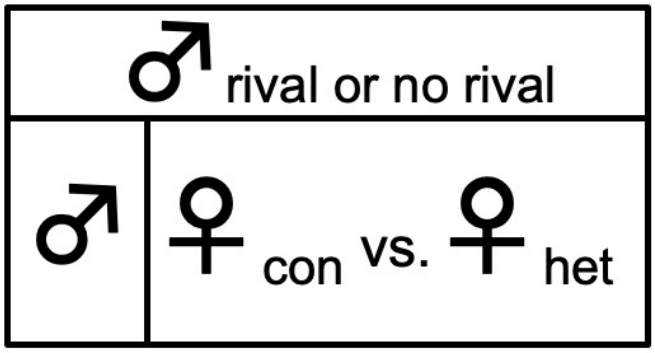

Figure 2.1
Male mate choice experimental tank set-up in the treatment with the presence of a rival male prior to mating trial.

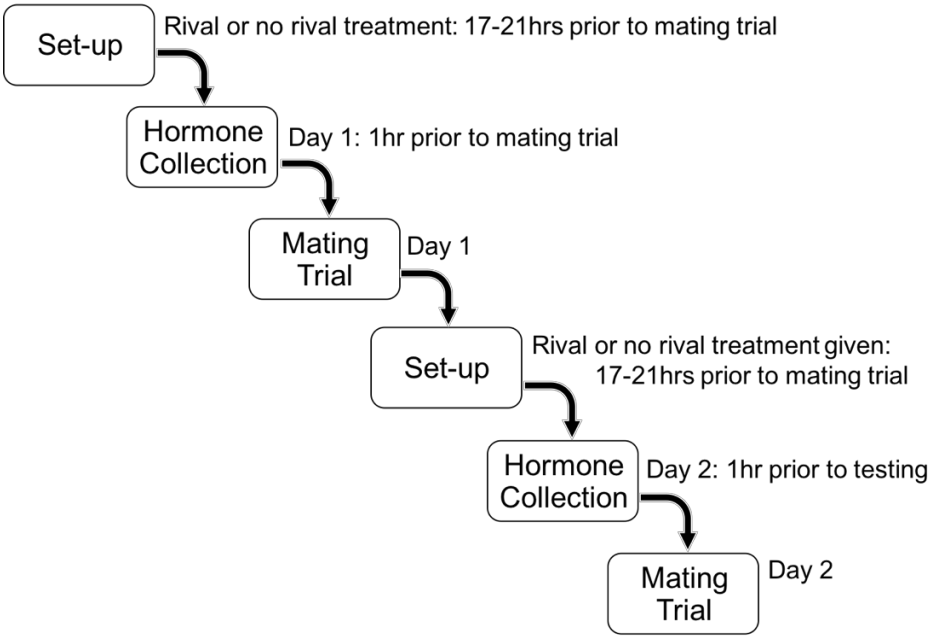

Figure 2.2
Summary of the experimental procedure for our repeated measures design.

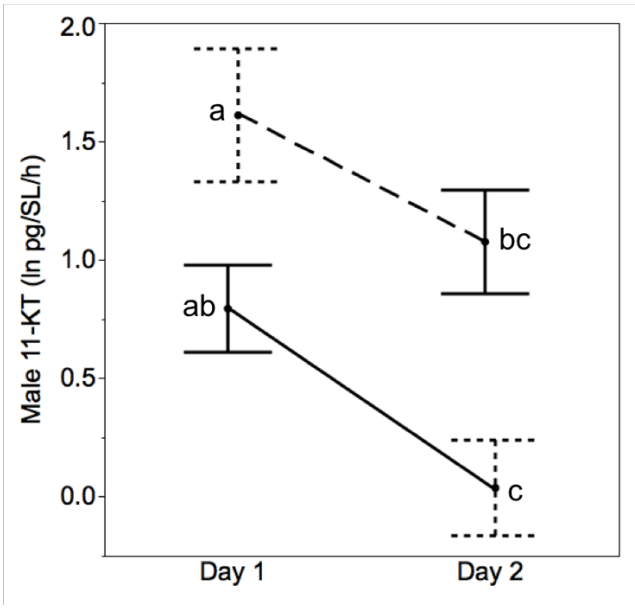

Figure 2.3
Mean ± S.E. of male 11-KT release rates by rival treatment (no rival: dashed error bars; rival: solid error bar) and by treatment order (no rival presented on first day: dashed line; rival presented on first day: solid line). Ln-transformed data are shown. Post-hoc comparisons show grouping by lowercase letters. Male 11-KT release rates of either treatment order decreased by the second day. However, male 11-KT release rates in the absence of a rival were lower on the second day after exposure to a rival on the first day.

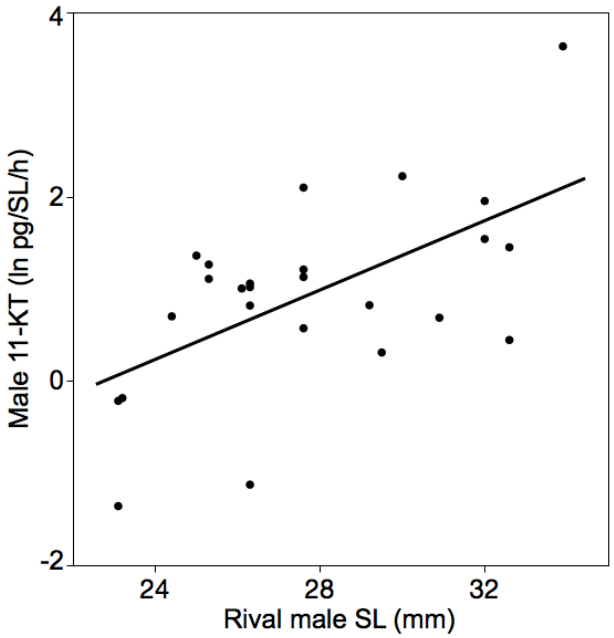

Figure 2.4
Correlation between male 11-KT release rates and the SL of the rival male. Ln-transformed data are shown.

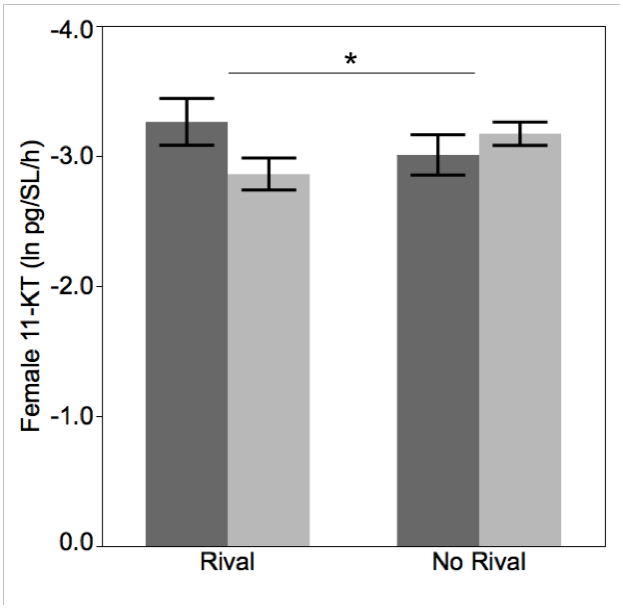

Figure 2.5
Mean ± S.E. of female 11-KT release rates by rival treatment and by female species (conspecific: dark gray bars; heterospecific: light gray bars). Ln-transformed data are shown. * indicates significant difference ($p < 0.05$) between rival treatments. There was no significant difference between species and no species by treatment interaction.

III. INDIVIDUAL VARIATION IN ACTH-INDUCED CORTISOL LEVELS IN FEMALES OF LIVEBEARING FISH AT DIFFERENT GESTATIONAL STAGES

Individuals vary in their baseline levels of stress hormones (predictive homeostasis) and in their stress responses (reactive homeostasis). Variation in normal reactive scope, both predictive and reactive homeostasis, may be important for understanding how endocrine traits respond to selection. Reactive homeostasis is the increase in glucocorticoid (GCs) hormones above baseline. Individuals at different life history stages, such as gestation in females, may show variation in normal reactive scope. We performed an adrenocorticotropic hormone (ACTH) challenge and measured changes in circulating GCs to estimate the reactive range of female sailfin mollies (*Poecilia latipinna*) at different gestational states. We measured cortisol, primary GC in teleost fishes, to obtain baseline release rates prior to injection with either ACTH or saline control. Using water-borne hormones, we measured cortisol release rates at four time intervals post-injection. Females were then sacrificed to determine the developmental stage of embryos, if present, and the number of developing embryos or mature ova. We found that ACTH-injected females had significant increases in cortisol releases rates, whereas cortisol release rates of control females did not change during the 4 hour post-injection period. We found high repeatability in predictive homeostasis of cortisol and moderate repeatability in reactive homeostasis and a phenotypic correlation between predictive and reactive homeostasis. Gestational state did not affect female predictive or reactive homeostasis. We applied the reactive scope model to *P. latipinna* and gained a further understanding of how among- and within-individual variation in both predictive

and reactive homeostasis are partitioned and how these traits vary under certain life-history conditions.

Introduction

Endocrine systems are highly variable within populations and across contexts (e.g. circadian and seasonal differences, age-dependence, life-history stages), and we are beginning to understand the extent to which endocrine traits vary among individuals (Williams 2008, Biro and Stamps 2015, Cox et al. 2016). Hormones are important for coordinating multiple facets of the phenotype including physiology, behavior, life history, and morphology (Taff and Vitousek 2016). Therefore, understanding individual differences in hormone responses can be informative about adaptation and the evolution of complex traits, such as the stress response (Ketterson and Nolan 1999, Dufty et al. 2002, Zera et al. 2007, Hau et al. 2016). The stress response promotes immediate survival through mobilizing energy stores, often at the expense of other life history traits such as reproduction (Sapolsky et al. 2000, Wingfield and Sapolsky 2003). The stress response may also show some degree of heritability (Cox et al. 2016). As a trait that shows variation among individuals and is heritable, the stress response can respond to selection in ways that maximize fitness benefits.

The stress response is a complex physiological mechanism that regulates an organism's response to perturbations and typically is measured through changes in glucocorticoid (GC) hormones before and after exposure to an aversive stimulus. The GC stress response is mediated through the HPA/I-axis (hypothalamus-pituitary-adrenal/interrenal) and GCs are the signaling hormones for target tissues in this physiological pathway (Wendelaar Bonga 1997). Exposure to stressors initiate the release of CRH (corticotropin-releasing hormone) from the hypothalamus, which in turn induces the release of ACTH (adrenocorticotropic hormone) from the anterior pituitary. ACTH

activates glucocorticoid synthesis, which then produces several physiological responses to cope with stressors. Romero et al. (2009) proposed the reactive scope model as an explanatory, graphical model that integrates homeostasis and allostasis to describe the stress response. According to the reactive scope model, individuals vary in some physiological mediator, such as GC levels (we use cortisol for this study), at both baseline levels (predictive homeostasis) and at increases above baseline levels in response to unpredictable events (reactive homeostasis). Predictive homeostasis varies according to life-history demands and therefore encompass circadian variation for seasonal and non-seasonal species. Species may show seasonal variation that corresponds to breeding or gestation events or species may show non-seasonal patterns (little to no variation across seasons) in predictive homestasis (Romero 2002). Any exposure to a stressor will drive increases in cortisol into the reactive range, but in healthy individuals, levels should rapidly return to baseline after the stressor has ended to maintain homeostasis. Combined, both predictive and reactive homeostasis constitute the normal reactive scope. Below the normal reactive scope is homeostatic failure, in which levels of cortisol are too low to maintain homeostasis. Any further increases in cortisol beyond the normal reactive scope is homeostatic overload, which can result in reduced immune function, suppressed reproduction, and decreased growth (McEwen and Wingfield 2003, Romero 2004, DuRant et al. 2016). The threshold for homeostatic overload presumably does not vary with circadian or circannual rhythms but may be reduced when an individual experiences frequent, chronic exposure to stressful events (e.g., Narayan et al. 2015).

The reactive scope model accounts for some among-individual variation in

predictive homeostasis but the extent to which among- and within-individual differences explain variation in the normal reactive scope is not consistent across taxa, nor across study conditions (Hau et al. 2016, Taff and Vitousek 2016). Calculating repeatability of traits is a useful estimate to understand how among- and within-individual phenotypic variance is partitioned. However, studies on the repeatability of stress hormone titers do not show consistent patterns with respect to either the predictive or reactive ranges. Greater within-individual variation than among-individual variation (low repeatability) in predictive ranges has been shown in birds (house sparrows, Passer domesticus, Romero and Reed 2008, e.g. great tits, Parus major, Baugh et al. 2014), and fish (e.g. largemouth bass, Micropterus salmoides, Cook et al. 2011). Conversely, high repeatability in predictive ranges has also been shown in other bird species (e.g. Florida scrub-jay, Aphelcoma coerulescens, Rensel and Schoech 2011) and amphibians (e.g. Fijian ground frog, Platymantis vitiana, Narayan et al. 2013, Narayan and Hero 2013). In contrast to the mixed results of predictive ranges, reactive ranges shows more consistent patterns of high repeatability (largemouth bass, Micropterus salmoides: Cook et al. 2011, Florida scrub-jay, Aphelcoma coerulescens: Rensel and Schoech 2011, Fijian ground frog, Platymantis vitiana: Narayan et al. 2013, Narayan and Hero 2013), but see Baugh et al. (2014) for an example of no repeatability of reactive homeostasis.

Additionally, there may be some correlation between predictive and reactive ranges within individuals. Predictive and reactive ranges can be positively correlated (e.g., individuals with high predictive values have high reactive values) as was found in great tits, *Parus major*, exposed to acute handling stress (Baugh et al. 2014), and predictive and reactive ranges can also be negatively correlated (e.g., individuals with

higher predictive values have a constrained reactive value) as was found in Fijian ground frogs, *Platymantis vitiana* (Narayan et al. 2013). The direction of the correlation between predictive and reactive ranges may provide insight into the flexibility of the homeostatic overload threshold. If the homeostatic overload threshold is fixed, then individuals with high predictive values may show reduced or constrained reactive values. Conversely, a flexible homeostatic overload threshold may allow both predictive and reactive values to show correlated increases in the presence of stressors. Measuring repeatability of both the predictive and reactive ranges provides the upper limit to the heritability of these endocrine traits and therefore provides information about the extent to which the stress response can evolve (Bonier and Martin 2016, Cox et al. 2016, Hau et al. 2016).

Gestation is likely to be a major source of circannual variation observed in the predictive range of cortisol in seasonal species (Romero 2002, Wingfield and Sapolsky 2003). Reproduction requires considerable energetic investment (Stearns 1992) and increases in stress hormones during this period may aid females by facilitating access to energy stores such as increasing blood glucose levels, breakdown of lipids, and inhibition of protein synthesis (Sapolsky et al. 2000). Therefore, gestating females may have greater predictive values of cortisol than non-gestating females (Romero 2002). For example in female Fijian ground frogs, *Platymantis vitiana*, both predictive and reactive corticosterone (primary GC in amphibians and reptiles) values were higher in vitellogenic females than in non-vitellogenic females (Narayan and Hero 2013). However, gravid female tuatara, *Sphenodon punctatus*, had greater baseline corticosterone and a dampened corticosterone response compared to non-gravid females (Anderson et al. 2014). Gestational stage of developing embryos may also affect circulating stress hormones of

females, particularly at later stages of development. In some mammals, females show increases in cortisol just prior to parturition (Cavigelli 1999, Pavitt et al. 2016). In addition, females with larger brood sizes may have greater reproductive effort, hence greater energetic investment, and may also show greater levels of stress hormones (Algera et al. 2017). Therefore, understanding how predictive and reactive ranges of cortisol differ within a species based on breeding phenology can identify different sources of variation in the normal reactive scope.

In this study, we estimate the parameters of the reactive scope model as proposed by Romero et al. (2009), using changes in cortisol as the physiological mediator. First, we test the hypothesis that there is a correlation between predictive (nominal baseline) and reactive (stress response) homeostasis of cortisol, regardless of within- and among-individual variation in these ranges. We also test the hypothesis that baseline and stress response ranges of cortisol in individual females will correlate with reproductive status. We predict that as females progress in their gestational state, baseline should increase. Similarly, we predict that female baseline should increase as a function of increasing brood size. Additionally, stress response also may vary with gestation and brood size.

We tested the reactive scope model using female sailfin mollies, *Poecilia latipinna*. This species of livebearing fish typically carry broods for ~30 days and are mainly lecithotrophic, where embryos rely on yolk for nutrition rather than through maternal provisioning (i.e. placental nutrition, Pollux et al. 2014). *Poecilia latipinna* have a long breeding season, especially in constant temperature springs in southern temperate North America (Robinson et al. 2011) and females have multiple broods per year. Female and male *P. latipinna* form loose aggregations (shoals) of conspecific and heterospecific

individuals (Schlupp and Ryan 1996). There is no social structure or dominance hierarchies among females, but males exhibit alternative mating phenotypes based on male size (Snelson 1985, Ptacek and Travis 1996). To test our hypotheses, we performed an ACTH challenge, which should provide upper range estimates for the reactive homeostasis, on female *P. latipinna* at different stages of gestation and measured changes in cortisol, the primary GC of teleosts (Wendelaar Bonga 1997, Mommsen et al. 1999, Arterbery et al. 2010).

Materials and methods

Animal collection and housing

We collected *P. latipinna* (n ≈ 120) from Spring Lake, Hays County, Texas (29.89°N, 97.82°W) in January 2015 and brought them to laboratory facilities at Texas State University, San Marcos, TX. Mature males are easily distinguished from females by the presence of a gonopodium, a modified anal fin used as an intromittent sexual organ. Only mature females (SL >32 mm) were used in this study. Females from this population are gravid from March through August, although some females can be gravid year round (Robinson et al. 2011). We maintained female fish in several single-sex 40 L aquaria (~10 fish/tank) at a constant temperature (25°C) on 14:10h light-dark cycle with UV fluorescent lighting. We coordinated our hormone collection to minimize daily cortisol variation due to feeding, which contributes to peaks in cortisol levels. By placing fish on a daily feeding schedule, we could predict peaks in cortisol due to feeding and avoid additional error in our data. Therefore, we fed fish daily from 1600 – 1800 h. We fed fish food pellets (Purina AquaMax 200) and supplemented with live brine shrimp for every

feeding. Texas State University Institutional Animal Care and Use Committee approved all procedures in this study (Protocol #IACUC20151175).

Experimental design

During May 2015, we performed our ACTH challenge from 0800-1400 h. We stopped any further data collection 2 hours prior to feeding. We randomly assigned females to one of two treatments: 1) ACTH solution injection (adrenocorticotropic hormone porcine pituitary, Sigma A-6303; $n = 34$), prepared in Ringer's solution [dosage: 0.23 IU/g body weight], or 2) Ringer's-solution injection (control; $n = 15$). Ringer's solution for freshwater teleosts was prepared with 128.1 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 0.2 mM $NaHCO_3$ (Ogawa et al. 1973). We selected the ACTH dosage based on previous studies (Bshary et al. 2007, Felix et al. 2013). Prior to the injection treatments, we obtained "nominal" baseline cortisol (hereafter baseline) levels by placing females in 250 mL sterile glass beaker of 100 mL of de-chlorinated water for one hour (following methods of Gabor and Grober 2010). Immediately after obtaining a baseline sample, we injected females intraperitoneally along their ventral body cavity using a 31G needle (0.3 mL, 7.9 mm) with 20 μL of either ACTH solution or Ringer's saline solution. Experimenters were blind to treatment assignments of females. After injection, we obtained our first post-injection hormone sample (stress responses) using the water-borne hormone collection method described above. We repeated this procedure every consecutive hour to obtain three more post-injection water samples for a total of 4 post-treatment samples. We stored all hormone samples at -20°C until hormones could be assayed (Ellis et al. 2004). After obtaining our last post-injection sample, we sacrificed

females by immersion in an ice bath at 2-4°C (Wilson et al. 2009). We then measured standard length (SL) and severed the head at the brain stem to ensure that individuals were dead before we dissected the fish along the ventral abdominal wall. We removed any eggs or embryos from the abdominal cavity and scored the developmental stage of the eggs or embryos using Haynes' (1995) classification methods, and then counted the number of fully mature ova (stage 3+) or the number of developing embryos.

Hormone extraction and assay

We extracted hormones from water samples using Sep-Pak C18 columns (Waters Corp., Milford, MA) placed on a vacuum manifold, and we eluted hormones using methanol (following Gabor and Grober 2010). We then evaporated the eluted solvent using nitrogen gas. We resuspended the resulting hormone residue in 1 mL solution of 95% enzyme immunoassay (EIA) buffer (Cayman Chemical, Ann Arbor, MI) and 5% ethanol and vortexed the resuspended samples for 1 hour. We assayed hormones using cortisol EIA kits (Item #: 500360, Cayman Chemical). We strictly adhered to protocols provided by the manufacturer for duplicate samples on 96-well plates, which we read on a spectrophotometer at 405 nm (Powerwave XS, Bio Tek Instruments, Inc., Winooski, VT). Gabor & Contreras (2012) previously validated cortisol EIA kits to assay water-borne cortisol in the same population of *P. latipinna* and found a significant correlation between plasma cortisol and water-borne cortisol. The sensitivity of cortisol EIA plates was 14.26-41.59 pg/ml. We used a pooled sample from non-experimental *P. latipinna* as our control in quadruplicate on each of 8 experimental plates. Our inter-assay coefficient of variation for the pool sample was 15.7% and our intra-assay coefficients of variation

ranged from 3.4% - 13.5%.

Statistical analyses

We first obtained cortisol release rates as ng/mL/h and then multiplied by reconstitution volume (1 mL). We divided our hormone values by SL for each fish to control for individual differences in size to obtain hormone units as ng/SL/h. Our hormone data were then log-transformed to better fit the assumptions of parametric analyses. However, all figures are illustrated using non-transformed data. We conducted all analyses in R version 3.2.3 (R Core Development Team). We used a linear mixed model (LMM) to determine differences in cortisol release rates between control and ACTH-injected females across the sampling hours from baseline to 4 hours post-injection (R package nlme::lme). Our predictors in this model were treatment, sampling hours, and an interaction as fixed effects and a random effect including random intercepts and slopes for females across sampling hours.

We calculated an adjusted repeatability (r) with a linear mixed model (LMM) based approach using the Restricted Maximum Likelihood (REML) method (Nakagawa and Schielzeth 2010, Dingemanse and Dochtermann 2013). Repeatability provides the proportion of total variance that is explained by among-individual variation. However, by using an adjusted repeatability for LMM-based approaches, we control for confounding factors such as fixed and random effects that may affect how variance is partitioned (Nakagawa and Schielzeth 2010). We specified a univariate mixed model using an R package (rptR::rptGaussian) for control and ACTH-injected females separately. Previous studies have used LMM-based approaches to calculate repeatability (Ouyang et al. 2011,

Baugh et al. 2014). For control females, we calculated repeatability using the cortisol measurements across all time samples as an estimate for the repeatability of predictive homeostasis ('baseline'). We used cortisol release rates as our response variable, sampling hours (baseline to 4h) as a fixed effect, and female identity with a random intercept effect. For ACTH-injected females, we calculated repeatability using cortisol release rates as our response variable, sampling hours (baseline to 4h) as a fixed effect, and female identity across time as a random slopes and intercepts effect.

We used Pearson's correlation to investigate if baseline values of cortisol release rates in females were correlated with their stress response values. We used the sampling hour post-injection with the highest cortisol release rates as the stress response for each ACTH-injected female.

To determine the effect of gestational stage and brood size on baseline cortisol release rates, we used two linear models with cortisol release rates as the response variable. Baseline cortisol release rates of females in both treatments (ACTH and control) were pooled and used as the response variable because the baseline sample was obtained prior to treatment injections. To avoid collinearity between gestational stage and brood size, gestational stage was used as a predictor variable in the first model and brood size was used as a predictor variable in the second model. However, because brood size was also correlated with SL (larger females tend to carry more eggs or embryos), we controlled for body size by using residuals from a correlation between brood size and SL ($r^2 = 0.54$, $p < 0.001$). To investigate the effects of gestational stage and brood size on female stress response cortisol release rates, we used stress response cortisol release rates as the response variable in two linear models. Treatment, gestational stage and an

interaction were used as predictor variables in the first model and treatment, residuals of brood size corrected for body size, and an interaction were used as predictor variables in the second model.

Results

Cortisol release rates differed across sampling hour for ACTH-injected females (Table 1; Fig. 1). Average baseline values for female *P. latipinna* (one baseline value per individual in both ACTH- and Ringer's solution-injected treatments) was 0.74 ng/SL/h with a range of 0.11 ng/SL/h to 1.84 ng/SL/h. Post-hoc Tukey's multiple comparisons showed that control females did not differ in cortisol release rates across times from baseline to 3h post-injection (all pairwise comparisons show non-significant *p*-values), but differed significantly between 3h post injection and 4h post-injection (Tukey's: $p = 0.034$, Fig. 1). Cortisol levels differed significantly across times for ACTH-injected females, with peak cortisol levels at 2h post-injection (Fig. 1). In addition, cortisol release rates between control and ACTH females did not differ at baseline (Tukey's: $p = 1$), but did differ significantly at all sampling hours (Tukey's: $p < 0.001$), second sampling hour (Tukey's: $p < 0.001$), third sampling hour (Tukey's: $p < 0.001$), and fourth sampling hour (Tukey's: $p = 0.007$) post-injection.

Repeatability was high in the control treatment ($r = 0.42 \pm 0.14$, 95% CI [0.12, 0.65], Fig. 2a); 42% of the variation in predictive cortisol release rates were due to among-individual variation ($p < 0.001$). Repeatability of cortisol release rates was moderate in ACTH-injected females ($r = 0.31 \pm 0.10$, 95% CI [0.15, 0.53], $p < 0.001$, Fig. 2b), and lower than repeatability of control females.

There was a significant positive correlation between baseline (predictive) and stress response (reactive) natural-log transformed cortisol (i.e., the single highest value from 1h to 4h post-injection) of ACTH-injected females (Fig. 3).

Baseline and stress response cortisol release rates did not vary with the gestational stage of females or the number of fully mature ova/developing embryos (Table 2 and 3, Fig. 4).

Discussion

We estimated the parameters of the reactive scope model proposed by Romero et al. (2009) by measuring both baseline and ACTH-reactive cortisol levels of female *P. latipinna* across different gestational stages. The lack of difference in cortisol release rates across sampling hours among our control treatment show that handling and injection did not induce a stress response in our control females (similar to Gabor & Contreras 2012). Female *P. latipinna* injected with ACTH showed a significant increase in cortisol levels across time when compared to females injected with saline. Cortisol release rates of ACTH-injected females was highest two hours post-injection but each individual female showed variation in the timing of their peak cortisol and in the magnitude of the increase. Both zebrafish (*Danio rerio)*, and anthias (*Pseudanthias squamipinnis)*, mounted a stress response when challenged with ACTH, similar to the results of our study (Bshary et al. 2007, Felix et al. 2013).

Some studies on mammals have shown a relationship between reproductive status and changes in maternal GCs (Carr et al. 1981, Dorr et al. 1989, Cavigelli 1999, McLean and Smith 1999, Obel et al. 2005), but other studies have not observed any such

relationship. Pribbenow et al. (2014) found no measurable increases in cortisol during the peripartal period in two species of lynx (*Lynx spp.*) whereas Pavitt et al. (2016) only found increases in cortisol during the peripartal period in older female red deer, *Cervus elaphus*. In amphibians, Narayan & Hero (2013) found that vitellogenic females showed greater baseline and short-term corticosterone responses to handling and capture stress. In contrast, we found no such relationship between GCs and reproductive status in female *P. latipinna*. The reactive scope of *P. latipinna* fits the basic graphic model for non-seasonal species (Romero et al. 2009). As a species with a long breeding season (~9 months to a year), female *P. latipinna* may not show the same seasonal patterns as short-term or explosive breeding species. Therefore, female *P. latipinna* maintain more constant baseline GCs across time rather than changing their energetic investment for reproduction. Additionally, this population of fish live in a spring-fed stream with constant year-round temperatures and therefore inhabit a relatively stable environment where the need to rapidly respond to unpredictable and dynamic changes is reduced. Therefore, there may be no need to mobilize energy stores via cortisol production, which assist in a rapid response to homeostatic perturbations, during gestation compared to other species that encounter a more stochastic environment and have a shorter breeding season.

Female *P. latipinna* may also be better adapted to handle additional stressors during gestation because their cortisol levels are not elevated during reproduction compared to species that show seasonal reproduction and, often, seasonal patterns in their normal reactive scope. Future studies can focus on how the reactive scope of other livebearing fish varies with reproductive status and test whether species with seasonal

changes in their reactive scope can respond to additional stressors by increasing their cortisol levels. Other livebearing fish such as *Gambusia spp*. have a broad latitudinal distribution and therefore may show more seasonal reproductive patterns. Furthermore, our study used a cross-sectional experimental design to investigate the correlation between circulating hormones and gestational status but conducting a longitudinal study of cortisol release rates for individual females across the span of gestation may detect smaller scale individual patterns.

Repeatability sets an upper bound to estimates of trait heritability (Lessels and Boag 1987, Boake 1989). Our calculations of repeatability show that cortisol levels characterizing predictive homeostasis in female *P. latipinna* were repeatable, which suggests that there is likely to be a heritable component to predictive (baseline) levels of cortisol release rates. High repeatability also suggests that within-individual variation in predictive levels of circulating cortisol in *P. latipinna* is low enough across a short time period (5 consecutive hours) that using a single observation of hormone concentration may be representative of an individual's predictive level. In ACTH-injected females, individuals showed moderate repeatability in their induced reactive (stress response) levels of cortisol. However, there are some limitations to our repeatability estimates for ACTH-injected females. By design, each cortisol measurement after an ACTH-injection represents a distinct physiological state, which increases within-individual variation and decreases repeatability estimates, thereby underestimating our measures of repeatability for induced reactive levels of cortisol. A more accurate measurement of repeatability might be obtained by conducting our procedure (baseline plus several samplings after ACTH injection) multiple times for each individual, provided enough recovery time was

given between sessions. Such a protocol would likely increase repeatability estimates. Yet, despite our underestimated measure of repeatability, we nevertheless found moderate repeatability in reactive levels of cortisol.

There was a positive correlation between our baseline and stress response values of cortisol release rates (e.g., individuals with high predictive cortisol also have high reactive cortisol). One possible hypothesis for a positive correlation may be that our nominal baseline measures do not reflect absolute baseline value. If an individual experiences a stressful event prior to the initial measurement of cortisol, then our estimate of baseline values may be inflated. However, we took all necessary precautions to significantly reduce potential stressors. All females were acclimated to laboratory conditions several months prior to the experiment, fed at the same time of day, and were exposed to similar biotic and abiotic environments. Further, we saw no effect of handling on cortisol levels. An alternative hypothesis is that the homeostatic overload threshold also shows considerable among- and possibly within-individual variation. If the homeostatic overload threshold is fixed, increased baseline cortisol (predictive) would constrain the extent to which animals can respond to a stressor (reactive) without detrimental effects. If, however, the homeostatic overload threshold is labile, an individual with high predictive homeostasis may have a higher threshold (i.e. exhibit plasticity in the threshold) that allows for a greater reactive range.

A phenotypic correlation also suggests that these endocrine traits could have a coordinated response to selection, given enough among-individual variation and a genetic correlation between the predictive or reactive ranges of cortisol. Although there is a significant phenotypic correlation between predictive and reactive cortisol in our study,

the relationship is weak (15% of the variance in reactive values are explained by predictive values). Baugh et al. (2014) also found a phenotypic correlation between baseline corticosterone and stress-induced corticosterone in great tits and attributed this phenotypic correlation to a strong within-individual correlation. Regardless of the mechanism, however, the phenotypic correlation between predictive and reactive ranges suggests that these endocrine traits are not independent of each other, and that this correlated relationship should be considered in future studies.

Our study highlights a need to further explore the large amount of variation in the stress response among individuals but also the need to further understand within-individual variation. There may be important evolutionary consequences of hormonal phenotypes if individuals remain consistent in their stress response but variation among individuals remain high. If there is phenotypic plasticity in the stress response (i.e., physiological flexibility), then selection could act upon this plasticity rather than a static trait value. More importantly, there is a need to further understand how the stress response changes, if at all, at different life history stages. This may aid in understanding how endocrine traits of a given species might respond to selection and the fitness consequences of environmental stressors on both individuals and populations.

Tables and table legends

Table 3.1
The effects of treatment across sampling hours on female cortisol release rates.

x	Estimate	SE	*t*	*p*
Treatment	-0.230	0.220	-1.048	0.300
Sampling Hour	0.078	0.036	2.145	**0.034**
Treatment x Sampling Hour	-0.208	0.066	-3.141	**0.002**

Table 3.2
Effect of reproductive state on female predictive (baseline) cortisol release rates. **Brood size represents residuals of the standard length vs. brood size regression.*

	x	Estimate	SE	*t*	*p*
Model 1	Gestational stage	-0.03	0.03	-1.10	0.277
Model 2	Brood size*	0.004	0.02	0.16	0.871

Table 3.3
Effects of reproductive state on female reactive (stress response) cortisol release rates.
******Brood size represents residuals of the standard length vs. brood size regression.*

	x	Estimate	SE	*t*	*p*
Model 1	Treatment	-0.55	0.39	-1.41	0.165
	Gestational stage	-0.003	0.03	-0.12	0.909
	Treatment x Gestational stage	-0.05	0.05	-1.05	0.299
Model 2	Treatment	-1.03	0.19	-5.48	**< 0.001**
	Brood size*	-0.04	0.02	1.77	0.086
	Treatment x Brood size*	-0.04	0.04	-1.03	0.310

Figures and figure legends

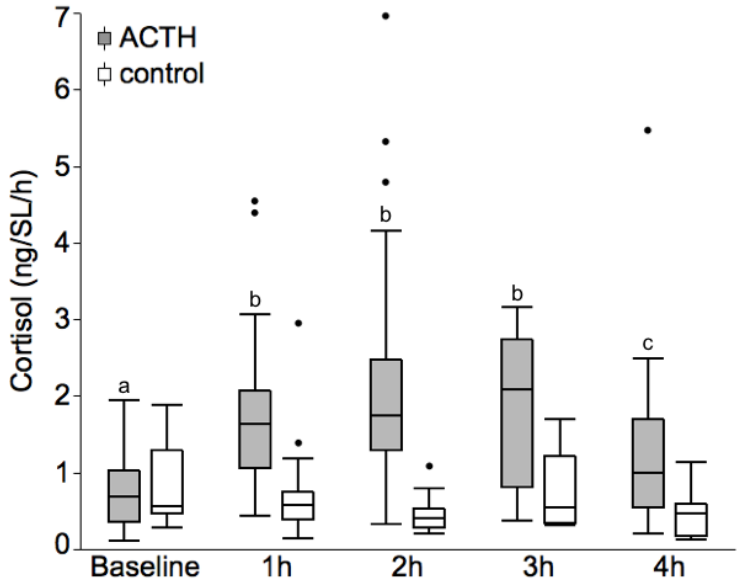

Figure 3.1
Cortisol release rates (ng/SL/h) of ACTH-injected and control-injected (Ringer's solution) female sailfin mollies, *P. latipinna*, across time from baseline to hour(s) post-injection. Cortisol release rates from control-injected females are not significantly different across time but cortisol release rates from ACTH-injected females are different across time. The lower and upper portion of the box indicate the 25% - 75% quantiles of each variable. The solid line indicates the median. The whiskers indicate the 90 and 10 percentiles. Single data points are outlying data points. Letters above ACTH columns indicate grouping of Tukey's test for ACTH treatment only.

a) b)

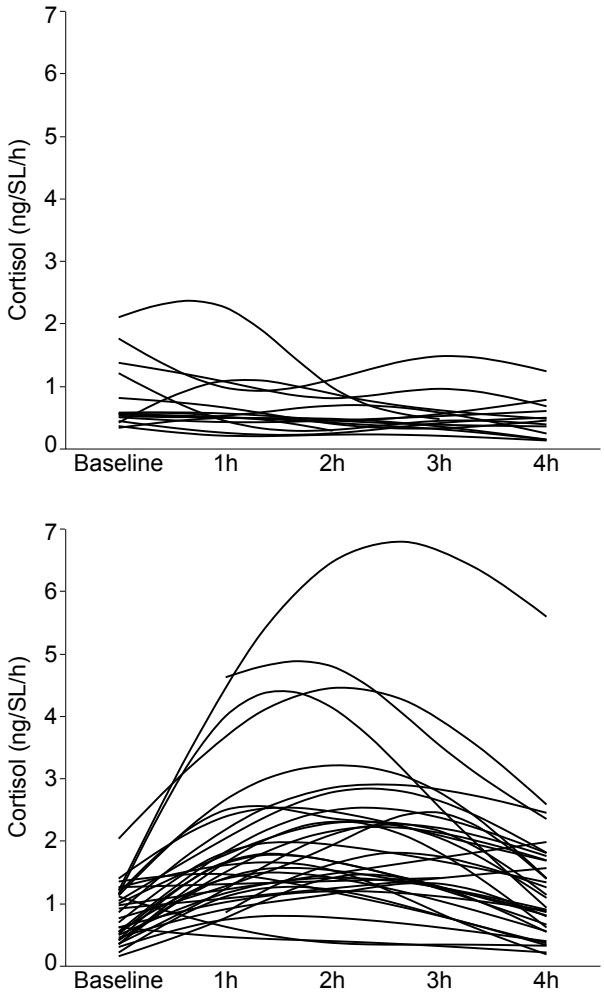

Figure 3.2
Individual variation in cortisol release rates (ng/SL/h) of a) control-injected (Ringer's solution) and b) ACTH-injected female *P. latipinna* across time from baseline to hour(s) post-injection.

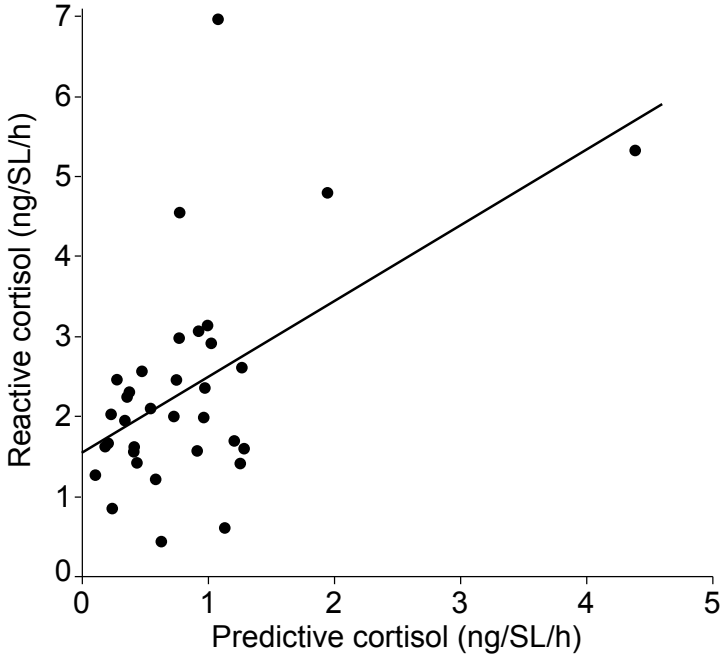

Figure 3.3
Correlation between predictive (baseline) cortisol release rates (ng/SL/h) and the highest reactive (stress response) cortisol release rate of individual ACTH-injected females (r^2 = 0.15, p = 0.024). Data in figure shows untransformed data, but all analyses were conducted with transformed data.

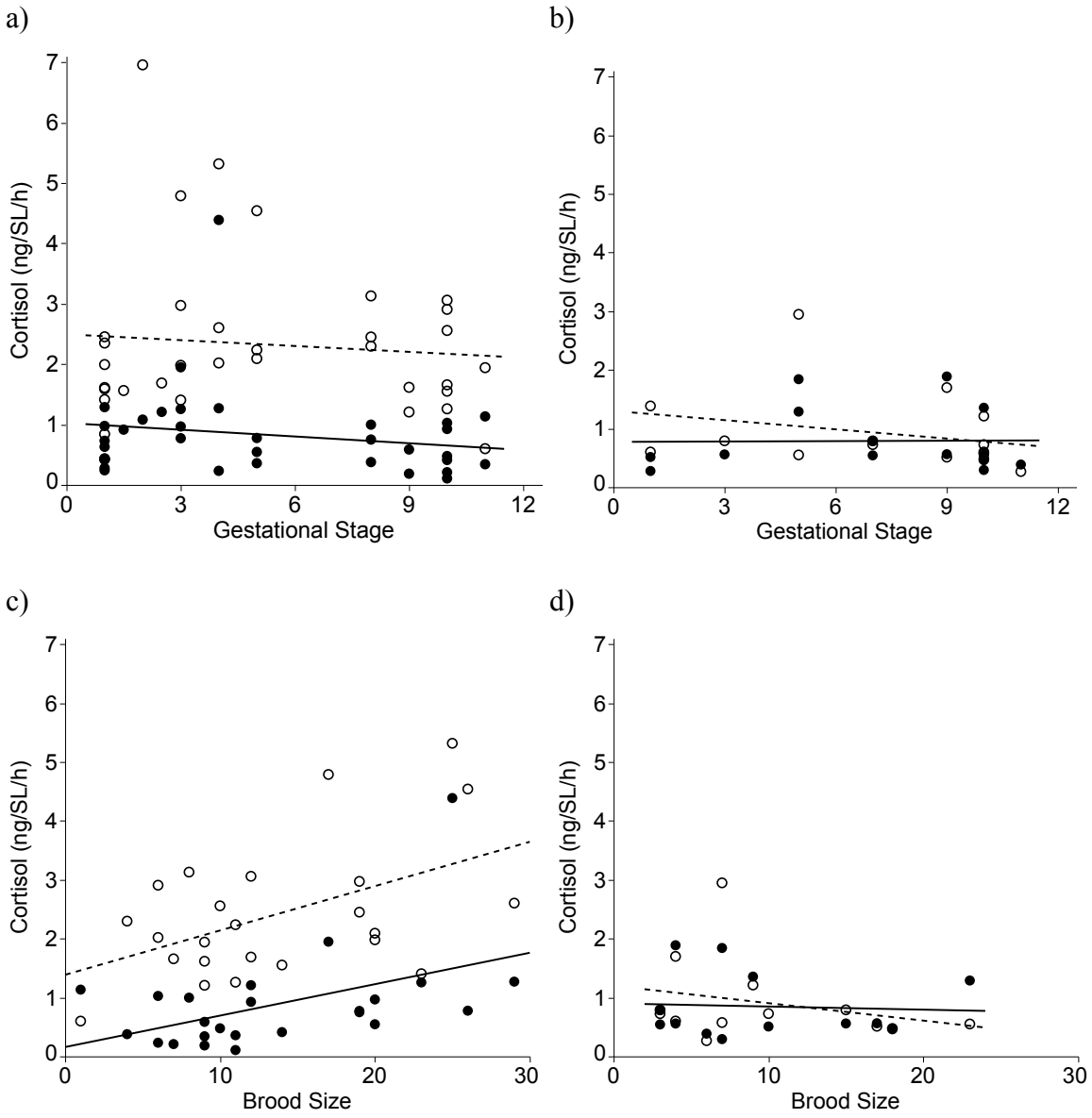

Figure 3.4
Cortisol release rates (ng/SL/h) of female *P. latipinna* predictive (baseline) range (black circles and solid line) and reactive (stress response) range (open circles and dashed line) with a) ACTH-injected females at different stages of gestation, b) control-injected females at different stages of gestation, c) ACTH-injected females carrying different brood sizes and d) control-injected females carrying different brood sizes.

IV. GENOME-WIDE ASSOCIATION STUDY SHOWS POLYGENIC BASIS OF PHENOTYPIC VARIATION IN MALE SAILFIN MOLLIES

A genome wide association study (GWAS) can contribute to our understanding of the genetic architecture of complex phenotypes and can be used to calculate heritability. Sexually-selected polymorphisms such as those found in alternative reproductive tactics (ARTs) are an example of complex phenotypes that show extreme variation among multiple traits. Males often show phenotypic traits that vary in size, ornamentation, coloration, and behavior. Simple genetic architectures are often predicted to favor the evolution of polymorphisms. Using male sailfin mollies, *Poecilia latipinna*, a live-bearing fish species, we investigated the genetic basis of male phenotypic variation. We characterize the underlying genomic architecture of relevant morphological traits that define ARTs in male sailfin such as body size and dorsal fin length. Using a Bayesian sparse linear mixed model approach, we found evidence that a large portion of variation in phenotypic traits were explained by genetic markers, suggesting high heritability estimates for these traits. Our data also suggests that ARTs in male sailfin mollies are polygenic and show complex genetic architecture with many loci of small effect and some loci of moderate effect contributing to phenotypic variation. This study indicates that sexually-selected polymorphisms may be maintained despite complex genetic architecture.

Introduction

Identifying the genetic architecture of complex phenotypes is important for estimating narrow-sense heritability, the proportion of phenotypic variation explained by additive genetic variance (Zaitlen and Kraft 2012). As a method to detect genetic architecture, researchers utilize genome-wide association studies (GWAS), a genomic mapping technique that tests the association between a genetic marker(s), such as a single nucleotide polymorphisms (SNPs), to variation in a phenotypic trait (Barsh et al. 2012). These association studies are powerful tools to characterize both simple genetic architecture, where one or few loci of major effect are detected, to complex architecture with many loci of small effect. Complex phenotypes include sexually-selected polymorphisms such as those found in alternative reproductive tactics (ARTs) which consist of a discrete suite of traits and behaviors (Oliveira et al. 2008b). Traits that vary among ARTs are likely to be targets of sexual selection, such as ornaments, colorations, and courtship behaviors.

A genetic basis to ARTs can be found in many taxa (Lank et al. 1995, Shuster and Sassaman 1997, Sinervo and Zamudio 2001, Ocana et al. 2014), including ARTs with polyphenisms (Moczek and Nijhout 2002). Additional sources of variation such as pleiotropic effects or gene by environment interaction (GxE) can contribute to phenotypic variation among ARTs (Tezuka et al. 2011, Carter et al. 2015). A simple genetic architecture should favor the persistence and maintenance of polymorphisms by limiting opportunities for recombination. However, a genomic association study conducted on ARTs in male Atlantic salmon (*Salmo salar*), showed that several genomic regions varied with phenotypes (Johnston et al. 2014). These associated regions were known to

contribute to various aspects of physiology, behavior, and morphology such as metabolism, immune response, mate choice, and muscle development. Quantitative traits such as body size, which is particularly important in the variation among different ART phenotypes, also appear to have complex genomic architecture (Gutierrez et al. 2015). Using genomic association studies in fish, researchers identified genomic regions responsible for body size and growth (Tsai et al. 2015, Yoshida et al. 2017, Li et al. 2018, Yu et al. 2018).

We investigated the genetic basis of complex phenotypes using the male sailfin molly (*Poecilia latipinna*), a live-bearing fish species. Male sailfin mollies are suited for studying complex phenotypes because males show large variation in morphology, coloration, and behavior that may have both genetic and environmental components to phenotypic expression. Body size shows a continuous distribution within populations (Travis 1994b, a). Variation in body size and courtship behavior in male sailfin mollies appears to have a genetic basis (Trexler and Travis 1990, Trexler et al. 1990, Travis 1994a, Ptacek 2002). However, intermediately-sized males of sailfin mollies show a great degree of plasticity in behavior depending on the relative size of males within the social environment (Travis and Woodward 1989, Fraser et al. 2014). Traits among male sailfin molly appear to be correlated and may covary with body size (Figure 1). Larger males typically exhibit exaggerated traits, striking coloration (yellow and black coloration), and perform courtship behaviors (Travis and Woodward 1989, Ptacek and Travis 1996). Conversely, smaller males do not have exaggerated secondary sexual characteristics or coloration and perform 'sneaking' behavior through forced copulations. Male body size varies according to the time it takes to sexual maturity, after which males do not continue

to grow (Snelson 1982, Travis et al. 1989), similar to that of other poeciliid species (Zimmerer 1982, Ryan et al. 1990, Ryan et al. 1992, Erbelding-Denk et al. 1994). Males also have a sail-like dorsal fin that scales to body size in a positive allometric relationship so that larger males have disproportionately larger dorsal fins (Farr et al. 1986, Hankison and Ptacek 2007). Males inseminate females internally with a modified anal fin, the gonopodium. Male gonopodia may scale in a negative allometric fashion to body size because sperm competition plays an important role among males of different sizes (Aspbury 2007). Therefore, smaller males likely have disproportionately larger gonopodium relative to their body size.

Prior studies have shown support for a simple genetic architecture of ARTs among poeciliids. Studies have shown that copy number variation of the mc4r gene (P-locus) is correlated with expression of ARTs based on body size in live-bearing fish of the genus *Xiphophorus* (swordtails and platyfish; Zimmerer and Kallman 1989, Lampert et al. 2010, Volff et al. 2013, Smith et al. 2015). Control of body size in *Xiphophorus* is an exemplary model of how one gene of large effect can result in extreme phenotypic variation. However, studies on sailfin molly species using interspecific cross found evidence for an additive polygenic genetic architecture to explain variation in morphological traits such as dorsal fin length (Ptacek 2002, Loveless et al. 2010). One limitation of association studies is the missing heritability problem, where heritability estimates in GWAS studies are typically lower than traditional methods used to estimate heritability such as those derived from family studies (Maher 2008, Eichler et al. 2010). (Yang et al. 2010). However, more robust methods of analyzing large genomic datasets have become available that addresses this missing heritability issue (Zhou et al. 2013).

Using a GWAS approach, we characterized phenotypic traits associated with ARTs in a sample of wild-caught male sailfin mollies. We selected body size, gonopodium length, dorsal fin height, and dorsal fin length as traits of interest to address the following questions: 1) Which phenotypic traits are correlated with variation in male body size and do these traits differ from females? 2) What are the heritability estimates for these male traits? Lastly, 3) what is the genetic architecture of these traits?

Methods

We caught male (n = 400) and female (n = 30) sailfin mollies from Spring Lake, Hays County, Texas (29.89°N, 97.82°W) using dip net and seining techniques (January 2015 - August 2017). All fish were transferred to laboratory facilities at Texas State University (San Marcos, TX). We housed fish in several 40 L single-sex aquaria (~5 male fish/tank or ~10 female fish/tank) at a constant temperature (25°C) on a 14:10 h light-dark cycle with UV fluorescent lighting. Mature male sailfin mollies were identified by the presence of a fully formed gonopodium. For photos, fish were placed in a custom-made holding tank with enough de-chlorinated tap water to cover the fish. Holding tanks were constructed so that the width of the tank restricted the movements of fish. Each fish was photographed against a 18% gray-card background and photographed with a ruler to standardize measurements across photos. Three or more pictures of each fish were taken on both sides with a Canon high-resolution digital camera. After photos were taken, we removed a small portion of the male caudal fin for genetic analysis. Fin samples were placed in 95% ethanol and stored at -80 °C until samples could be sequenced.

Statistical analysis

From pictures, we measured the following morphological traits in both males and females using the software ImageJ (http://rsbweb.nih.gov/ij/): standard length - length of the fish from the lip to the peduncle; anal fin length– length from the anal pore to the tip of the gonopodium of males or the anal fin length of females; dorsal fin height – length of the 5th dorsal fin ray (or equivalent) from base to tip; and dorsal fin length - length of the dorsal fin at the base of the fin (Figure 1). We used a ratio of trait size to body size for anal fin length, dorsal fin height and dorsal fin length to account for allometric relationships. An isometric scaling relationship indicates that trait size is proportional for body. Alternatively, positive scaling relationship indicates that the relative trait size increases with body size, so that individuals with larger body size have disproportionately larger trait values. Whereas individuals with disproportionately smaller trait value relative to body size would indicate a negative scaling relationship. To determine allometric relationships between traits, we used a linear regression between body size and anal fin length, dorsal fin height or dorsal fin length. All traits values were then z-transformed for all subsequent analyses. We used a principle component analysis (PCA) to characterize intra- and inter-sexual differences in phenotypic traits. If phenotypic traits vary between the sexes, then females should cluster tightly together in the ordination plot and males should fall out separately. If male phenotypes fall into two distinct tactics, then PCA scores for males should further separate out in two clusters.

DNA extraction and library preparation

We extracted genomic DNA from the fin clips of individual males (n = 354) using DNeasy Blood and Tissue Kits (Qiagen Inc. Valencia, CA), which consisted of males

across the range of phenotypic variation. We prepared a reduced representation genomic library for each individual by following previously established protocols (Parchman et al. 2012, Gompert et al. 2014, Sung et al. 2018). These protocols were also previously used on this species (Alberici da Barbiano et al. 2013). The genome Briefly, all genomic samples were first digested with two restriction endonucleases, *MseI* and *EcoRI* (New England Biolabs). We then ligated customized 8 – 10bp oligonucleotide barcodes that acted as unique individual identifiers as well as Illumina adaptor sequences to the resulting DNA fragments. We amplified these fragments through two rounds of polymerase chain reaction using Illumina PCR primers. All PCR products were pooled and sent off for size-selection (200-350bp length fragments) using Blue pippin technology and then for sequencing to the University of Texas Genomic Sequencing and Analysis Facility (Austin, TX). Samples were sequenced across two lanes on the Illumina HiSeq4000 platform generating 150 bp single-end reads.

After obtaining sequences, we first removed barcodes from each fragment using customized Perl scripts. There is no reference genome for *P. latipinna,* which has a genome size of ~815 million base pairs, therefore we performed a *de novo* assembly using a random subset of sequences using dDocent assembly. There is a reference genome for *P. formosa*, a closely-related hybrid species. However, aligning our sequences to this reference genome did not yield sufficient loci, and therefore we proceeded with a *de novo* assembly of our dataset. Sequences with at least 4 reads and sequences present in at least 4 individuals were included in construction of a reference scaffold. These filtered sequences were assembled to each other using an 80% similarity threshold. This generated a reference set of 51,965 scaffolds. We then aligned all parsed

reads to the consensus reference set of scaffolds using BWA (version 0.7.12-r1039) and allowed up to 6 bp mismatches. We used SAMTOOLS (version 1.2) and BCFTOOLS (version 1.2) to identify variant sites among assembled contigs (Li et al. 2009). In our BCFTOOLS settings, we used full priors, designated SNPs that were present in at least 70% of individuals, and the posterior probability that all samples were homozygous at the reference allele was <0.05. We then randomly selected a single SNP per contig to reduce non-independence among SNPs. We further removed individuals with low coverage (n = 8) with less than a mean of 0.5 reads per locus for n = 346 males. Our filtered dataset resulted in 27,304 variable sites.

Genome-wide association mapping

To understand the genetic architecture of alternative mating tactics in male sailfin mollies, we used a Bayesian sparse linear mixed model (BSLMM) approach with GEMMA version 0.98 (Zhou et al. 2013). This analysis is a hybrid approach which combines a linear mixed model (LMM) and a Bayesian variable selection regression model (BVSR), also known as sparse regression. The linear mixed model approach assumes a polygenic basis to phenotype and includes all variants in a model, whereas a Bayesian variable selection regression approach assumes only a small subset of variants affect phenotype (Guan and Stephens 2011). A BSLMM approach is a standard linear model which includes estimates of β, regression coefficients referred to as sparse effects and one random polygenic term. An MCMC algorithm allows us to estimate the proportion of phenotypic variance explained (PVE) by both sparse effects and polygenic effects, the proportion of genetic variance (PGE) explained by sparse effects, and the

number of variants with sparse effect (n). The BSLMM also generates a centered kinship matrix by calculating genetic relatedness from the genomic data. This accounts for any population stratification that may be present in the data when estimating effect sizes of individual SNPs. From the BSLMM, each SNP is assigned a posterior inclusion probability (PIP) to summarize the frequency a variant has a non-zero sparse effect in the MCMC. Sparse effect sizes were calculated as β x PIP. We investigated the genetic architecture of four traits (standard length, gonopodium length, dorsal fin height, and dorsal fin length) using GEMMA. We ran 5 MCMC chains with a 1,000,000 step burn-in, a length of 1,000,000 steps, and a thinning interval of 20.

Results

Gonopodium length, dorsal fin height, and dorsal fin length showed allometric relationships to body size (Figure 2). Larger males had a shorter gonopodium ($r2 = 0.465$, $p < 0.0001$), a taller dorsal fin ($r2 = 0.770$, $p < 0.0001$), and a longer dorsal fin ($r2 = 0.508$, $p < 0.0001$). Males and female morphological traits formed distinct clusters in a PCA plot of PC axis 1 (58.4% of variation explained) and PC axis 2 (33.3% of variance explained, Figure 2). PC axis 1 included high loadings for standard length and dorsal fin height, while PC axis 2 separated males and females from each other with high loadings for anal fin length and dorsal fin length (Table 1). However, there was greater variation among male phenotypes than females across PC axis 1. Additionally, males did not form discrete clusters in PC space, but rather showed continuous distribution across PC axis 1.

We detected strong evidence for a polygenic basis to phenotypic traits associated with ARTs in male sailfin mollies. Large PVEs (proportion of the phenotypic variance

explained) were detected for standard length, dorsal fin height, and dorsal fin length (Table 2). Although anal fin length had lower PVE than the other traits, anal fin length still showed that a moderate proportion of the phenotypic variance was explained by SNPs. In addition, the majority of the variance explained did not come from major effect loci (i.e. sparse effects) because estimates of PGE (proportion of genetic variance explained), which only includes sparse effects, for all four traits were relatively low (Table 2). There was also considerable uncertainty in our estimates of the number of variants with measureable effect (*n*). Estimates for *n* ranged from 40-99 variants (Table 2). However, sparse effect sizes (β x PIP) were small and spread across many SNPs for 3 out of 4 traits (Figure 4). We identified a single SNP that showed a significant association with dorsal fin height using a stringent threshold of PIP > 0.1 (Chaves et al. 2016).

Discussion

Using a genomic association study, we found that the phenotypic traits associated with ARTs in male sailfin mollies show a complex genetic architecture with many loci of small effect. Our results suggest that these ART traits are polygenic and high PVE values reflect high heritability. Three of the four phenotypic traits did not have any SNPs with large sparse effect size, but the cumulative effects of the sparse loci contributed a moderate amount to the proportion of genetic variance explained. In contrast, one locus associated with dorsal fin height showed major sparse effect size. Similarly, the majority of other association studies using the BSLMM approach that show a polygenic basis to a phenotypic trait also show some loci with major effect (Chaves et al. 2016, Lloyd-Jones et al. 2017, Lucas et al. 2018, Lundregan et al. 2018). The BSLMM approach to

association studies is a particularly useful tool to detect genetic architecture that consists of both many loci of small effect and few loci of large effect. However, the BSLMM approach can suffer from loss of power when sparse effects are underestimated and PVE is overestimated due to inclusion of many small effect loci. Although our data may be susceptible to overfitting, as with most GWAS datasets, our estimates of PVE show narrow credible intervals. The traits measured in our study show strong evidence of a polygenic basis and BSLMM is robust compared to other GWAS analysis methods for highly heritable traits (Lloyd-Jones et al. 2017). This study indicates that sexually-selected polymorphisms may be maintained despite complex genetic architecture.

We found that body size and dorsal fin height had higher PVE and lower PGE estimates than either gonopodium length or dorsal fin length. Complex traits such as body size, are likely to have complex genetic architecture because of variation in factors that determine body size such as environmental conditions, physiology, metabolism, and development (Johnston et al. 2014). If there is any single locus that determines body size in male sailfin mollies (e.g. mc4r gene/P-locus in *Xiphophorus*), we did not find any evidence of a monogenic basis to ARTs in sailfin mollies. We are cautious to conclude that there is not a singular locus that significantly contributes to male ARTs because we likely sampled only a small portion of the whole genome (*P. latipinna* genome size is ~815 million base pairs). However, our data suggest that a high proportion of variance in body size is attributed to many loci of small effects. In contrast, we found one candidate SNP that may be associated with a causal gene for dorsal fin height but not in other traits, which suggests that ART traits either have no major effect loci or there are very few. If traits such as gonopodium length or dorsal fin length have a similar genetic architecture

to dorsal fin height, where one major effect locus (or a few loci) contributes to a large portion of the variation explained, then we may have not sampled the relevant region of the genome with our current dataset of variants. In addition, the polygenic basis to trait architecture in our study may be due to high linkage disequilibrium. Although we filtered our dataset to reduce any tight physical linkage between variants, without a reference genome, we may not have completely accounted for linkage disequilibrium. Although there is a reference genome for a closely related hybrid species, *Poecilia formosa* (GenBank Assembly ID: GCA_000485575.1), aligning our data to this reference genome did not yield sufficient loci. In a previous study, Loveless et al. (2010) show species differences among mollies in both dorsal fin size and gonopodium length. This breeding study using F1 hybrids and backcrosses, had lower estimates for the proportion of trait variance explained by additive genetic variance compared to our study for dorsal fin length (Loveless et al. 2010:17%, this study: 70%), dorsal height (Loveless et al. 2010: 41%, this study: 80%), and gonopodium length (Loveless et al. 2010: 6%, this study: 41%). These differences highlight the importance of a GWAS approach to understanding genetic architecture of phenotypic variance.

In our current study, there was some overlap between SNPs with the highest sparse effect sizes across traits. One SNP was shared between standard length and anal fin length, and another SNP was shared between standard length and dorsal fin height, which suggest a shared genetic basis. Some phenotypic covariance between traits may be due to pleiotropic effects. Studies have shown that the same genomic regions may affect more than one trait (Endler et al. 2018). In *Philomachus pugnax*, differences between male ARTs, where morphs vary in a suite of morphological, physiological, and

behavioral traits, are determined by one supergene (Kupper et al. 2016).

Overall, we found a highly heritable, polygenic basis to male morphological traits associated with ARTs in sailfin mollies. We also detected some sparse effects and were only able to identify one significant variant in dorsal fin height. Using a hybrid BSLMM approach to characterize the genetic architecture of sexually-selected traits allowed us to capture the effects of many small effect loci and few moderate effect loci that may have been overlooked in other analyses, while still accounting for possible population structure (or more likely cryptic relatedness in our case). Finding a complex genetic architecture in male ARTs in our study contrasts with the prediction that simple genetic architectures favors the evolution of polymorphisms, but nonetheless contributes to our understanding of the evolution and maintenance of ARTs. Future studies on ARTs can focus on how complex phenotypes with a polygenic basis are maintained when faced with recombination.

Tables and table legends

Table 4.1
PCA loading matrix for phenotypic traits in both male and female *P. latipinna*.

	PC I	PC II
Standard Length	0.873	-0.392
Anal Fin Length	-0.524	0.811
Dorsal Fin Height	0.883	0.360
Dorsal Fin Length	0.721	0.624

Table 4.2
Mean of parameter and hyper-parameter estimates with 95% credible intervals [ETPI] for proportion of phenotype variance in male sailfin mollies explained by both sparse and polygenic effects (PVE), proportion of genetic variance explained by sparse effects (PGE), the number of variants with sparse effects (n), and the mean sparse effect size (β * PIP).

	PVE	**PGE**	n	**Effect size (β * PIP)**
Standard length	0.85 [0.78, 0.98]	0.23 [0.05, 0.35]	39.8 [5, 57]	0.0001 [0.00002, 0.0005]
Anal fin length	0.41 [0.21, 0.58]	0.36 [0.11, 0.57]	98.6 [14, 167]	0.00007 [0.00002, 0.0003]
Dorsal fin height	0.80 [0.70, 0.96]	0.26 [0.06, 0.39]	57.6 [7, 76]	0.0002 [0.00003, 0.0007]
Dorsal fin length	0.70 [0.54, 0.91]	0.29 [0.07, 0.45]	79.8 [10, 130]	0.0001 [0.00003, 0.0004]

Figures and figure legends

Figure 4.1
Photograph of the extreme traits in phenotypic variation of male sailfin mollies (*Poecilia latipinna)*. A) Standard length B) Anal fin length C) Dorsal fin height D) Dorsal fin length.

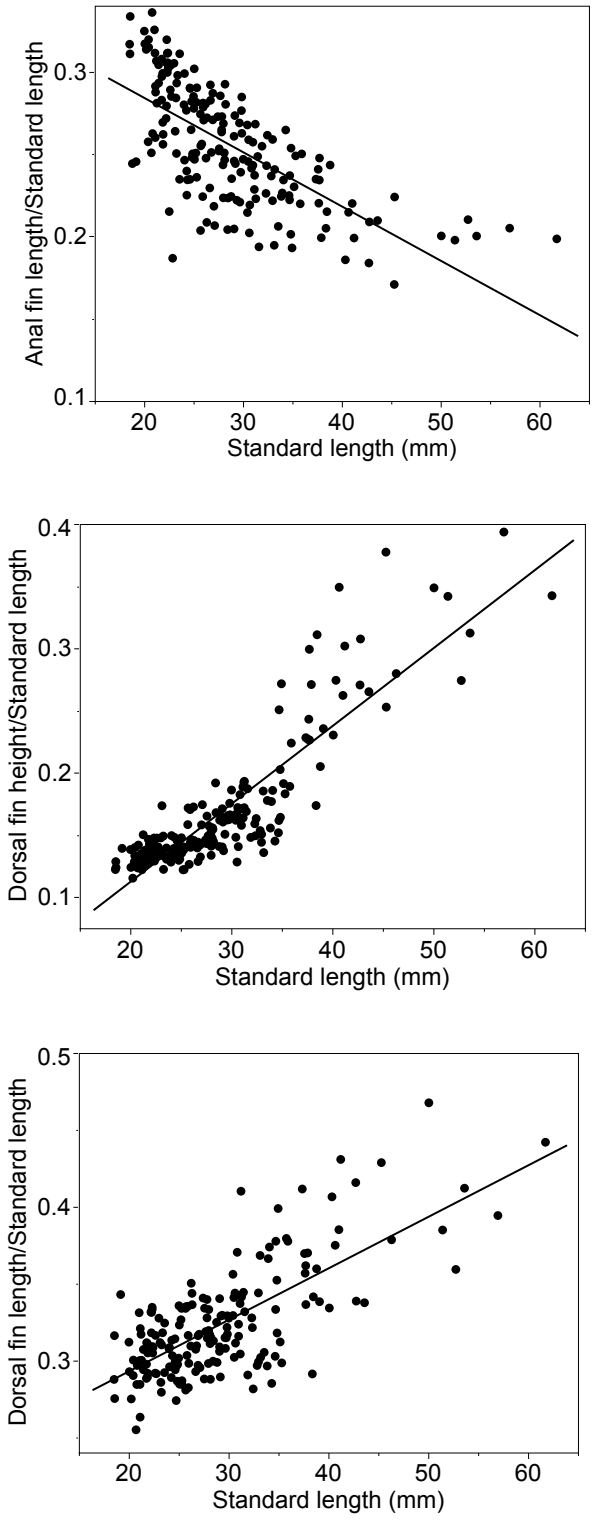

Figure 4.2
Linear regression between standard length and anal fin length; dorsal fin height, and dorsal fin length for sailfin mollies.

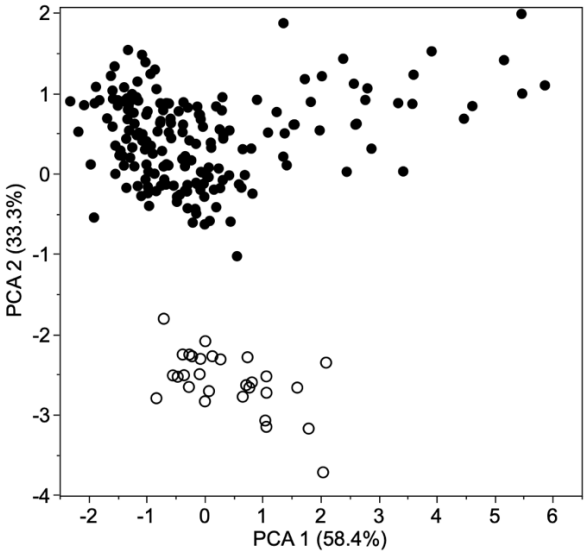

Figure 4.3
Principal components analysis of morphological variation in sailfin mollies. Open circles are females and filled circles are males.

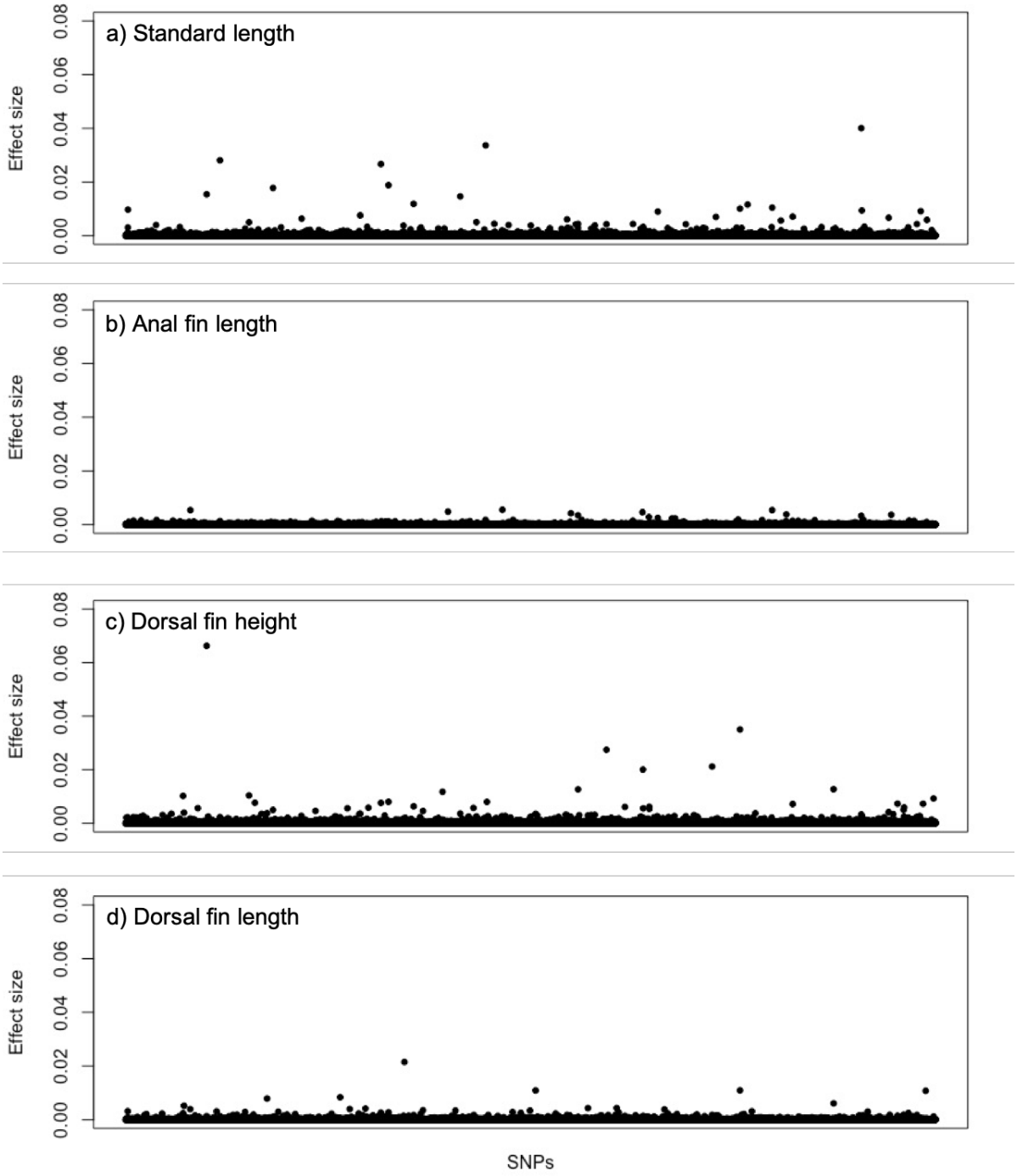

Figure 4.4
Manhattan plot of effect size (β * PIP) of each SNPs for sailfin mollies.

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