

PROXIMATE AND ULTIMATE PERSPECTIVES ON THE MAINTENANCE OF A
UNISEXUAL-BISEXUAL MATING COMPLEX: IMPORTANCE
OF BEHAVIORAL SYNDROMES AND
STRESS HORMONES

THESIS

Presented to the Graduate Council of
Texas State University-San Marcos
in Partial Fulfillment
of the Requirements

for the Degree

Master of SCIENCE

by

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San Marcos, Texas
August 2012

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ACKNOWLEDGEMENTS

I am extremely grateful to everyone that has contributed to the development and successful completion of this research. First and foremost, I thank my research advisor, Dr. Caitlin Gabor. She has been a great source of inspiration and a great mentor during my time here at Texas State University-San Marcos. For this, I am extremely grateful. I also thank my committee members, Chris Nice and Andrea Aspbury, for their guidance throughout this project, especially in times when Caitlin and I needed a fresh perspective on the project. Additionally, I thank Andrea Aspbury and Floyd Weckerly for invaluable help with statistical analyses. I especially thank Laura Alberici da Barbiano, Chelsea Blake, Adam Contreras, and the army of undergraduates for their great assistance with catching and maintaining fish, which made this project possible. Furthermore, I thank the members of the GASP lab, both past and present, for their support and intellectual contributions to this project. I especially am thankful for the friendship of Laura Alberici da Barbiano, Drew Davis, and Lily Swanbrow Becker, who truly made these past two years unforgettable. I thank my undergraduate advisor, Ron Mumme, for his continued support of me. I truly thank Shannon Fetcko for her unwavering support of me these past few years and for all of the sacrifices she has made in order to make this graduate experience possible. Finally, I thank my parents, my brother, and my wonderful friends from back home for their everlasting encouragement, and most importantly, for reminding me that life is not all about work and should not be taken so seriously.

This manuscript was submitted on May 17, 2012.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	v
LIST OF TABLES	viii
LIST OF FIGURES	ix
ABSTRACT	x
 CHAPTER	
I. MATING SYSTEMS, SEXUAL SELECTION, AND HORMONES	1
Natural History of the Unisexual-Bisexual Mating Complex	1
Sexual Selection and Species Recognition	3
Behavioral Syndromes	5
Hormones and Behavior	8
Rationale and Objectives	11
References	12
 II. INDIVIDUAL VARIATION IN MALE BEHAVIORAL TYPE AND ITS CORRELATES WITH MALE MATE PREFERENCE AND STRESS	
HORMONES	21
Abstract	21
Introduction	22
Methods	26
Fish Maintenance	26
Identification of Behavioral Types	26
Statistical Analysis	30
Results	31
Identification of Behavioral Syndromes	31
Repeatability of Behaviors	31
Behavioral Syndromes and Mate Choice	32
Behavioral Syndromes and Stress Hormones	32
Discussion	33
Evidence for Behavioral Syndromes	33
Behavioral Syndromes and Mate Preference	34

Behavioral Syndromes and Hormones.....	36
Conclusions.....	38
Acknowledgements.....	39
Tables.....	40
Figures.....	42
References.....	43

III. EXAMINATION OF CORRELATED BEHAVIORAL TRAITS AND STRESS HORMONE PRODUCTION BETWEEN TWO SPECIES IN A UNISEXUAL-BISEXUAL MATING COMPLEX	48
Abstract.....	48
Introduction.....	49
Methods.....	54
Fish Maintenance	54
Identification of Behavioral Types	55
Hormone Sampling	56
Data Analysis	57
Results.....	60
Identification of Behavioral Syndromes	60
Repeatability of Behaviors.....	60
Behavioral Syndrome Comparison between Species	60
Behavioral Syndromes and Hormones.....	61
Discussion	61
Evidence for Behavioral Syndromes	62
Species-level Differences in Behavioral Syndromes	63
Stress Hormones and Behavioral Syndromes	65
Conclusions.....	67
Acknowledgements.....	68
Tables.....	69
Figures.....	71
References.....	73

LIST OF TABLES

Table	Page
1. Spearman's rank correlations among the five behavioral categories that were measured, also including standard length (SL) and male strength of preference for conspecifics (SOP).....	40
2. Component loadings of behaviors onto two, orthogonal principal components and the variance explained by each.	40
3. Individual consistency (Spearman's rank correlation) and repeatability (intraclass correlation coefficient; ICC) of the three behaviors measured in Treatment 1 over two days.....	41
4. Spearman's rank correlations between the behavioral contexts associated with Treatment 1, as well as individual component scores (PC1), and baseline stress hormone release rates.	41
5. Spearman's rank correlation for each of the pair-wise behavioral comparisons between species.	69
6. Component loadings of behaviors onto principal components, as well as the variance explained by each component.	69
7. Repeatability (intraclass correlation coefficient; ICC) of three behaviors over a two-day time period for each species.....	69
8. Spearman's rank correlation between individual behavioral metrics, composite behavioral metrics (PC scores) and measures of stress hormone (cortisol) production for each species.	70

LIST OF FIGURES

Figure	Page
1. Number of gonopodial thrusts (\pm SE) by male sailfin mollies (n=50) to heterospecific (<i>P. formosa</i>) and conspecific (<i>P. latipinna</i>) females.....	42
2. Box plot representing baseline cortisol release rates (pg SL ⁻¹ hour ⁻¹ ; median \pm 1 st and 3 rd quartiles) in males which did (YES; gray) or did not (NO; white) exit the acclimation chamber in Treatment 1.	42
3. Biplot of the variable scores extracted from the first two principal components for each species.....	71
4. Difference in the proportion of individual behavioral types between species based on their PC1 score.	71
5. Average cortisol release rates (pg g ⁻¹ hour ⁻¹) for each species.	72
6. Difference in the cortisol response between Amazon mollies (<i>P. formosa</i>) and female sailfin mollies (<i>P. latipinna</i>).	72

ABSTRACT

PROXIMATE AND ULTIMATE PERSPECTIVES ON THE MAINTENANCE OF A UNISEXUAL-BISEXUAL MATING COMPLEX: IMPORTANCE OF BEHAVIORAL SYNDROMES AND STRESS HORMONES

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August 2012

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The persistence of gynogenetic organisms is an evolutionary paradox. An ideal system for examining the persistence of gynogens is the unisexual-bisexual mating complex of the unisexual Amazon molly (*Poecilia formosa*), and the bisexual, host species, the sailfin molly (*P. latipinna*) and the Atlantic molly (*P. mexicana*). Insight into the maintenance of this mating complex might be enhanced by taking a more holistic view of male and female behavior through a behavioral syndrome framework. Studies of

behavioral syndromes examine individual consistency and repeatability through time in suites of correlated behavioral traits. In this study, I examined whether male sailfin mollies (n=50) exhibited behavioral syndromes (BS) and if there was a relationship between BS and male mate preference as well as stress hormone production.

Additionally, I examined whether female sailfin mollies and Amazon mollies (n=40 of each species) exhibited behavioral syndromes. Furthermore, I examined whether there were differences in BS across species, whether there was a relationship between BS and stress hormone production, and whether there was a difference in stress hormone production across species. Male sailfin mollies exhibited a BS, with multiple correlations among activity, boldness and exploration traits, as well as correlations among activity and social traits. I did not find a significant relationship between BS in males and male mate preference; however, there was a relationship between boldness and baseline cortisol production. I did not detect a significant difference in BS between female sailfin mollies or Amazon mollies, although I found a relationship between the stress response and boldness and explorative behaviors, as well as a difference in stress hormone production between species. Taken together, these results suggest that there is a proximate link between BS and stress hormones; however, the ultimate relationship between BS and the maintenance of the unisexual-bisexual mating complex is less clear.

CHAPTER I

MATING SYSTEMS, SEXUAL SELECTION, AND HORMONES

Natural History of the Unisexual-Bisexual Mating Complex

Amazon mollies, *Poecilia formosa*, are a clonal, all-female species of live-bearing fish of hybrid origin (Hubbs & Hubbs 1932; Dawley 1989). Amazon mollies reproduce via gynogenesis, a particular form of asexual reproduction. Gynogenetic organisms require sperm to induce egg development, but paternal genetic information is not incorporated into the genome of the offspring (Hubbs & Hubbs 1946; Kallman 1962; Schultz 1967). Amazon mollies originated approximately 100,000 years ago and require sperm from one of their parent species, either sailfin mollies (*P. latipinna*) or Atlantic mollies (*P. mexicana*), to start embryogenesis, but none of the male genes are incorporated into the offspring (Avisé et al. 1991; Scharl et al. 1995; Stöck et al. 2010). As such, Amazon mollies are considered sexual parasites of the host (i.e. parent) species.

The Amazon molly is found in brackish water habitats from drainages in south Texas to the Rio Tuxpan in northern Mexico (Darnell & Abramoff 1968; Miller 1983; Schlupp et al. 2002). Sailfin mollies can be found coastally from North Carolina and through the Gulf of Mexico Coast south to the Rio Tuxpan in northern Mexico (Miller

1983; Page & Burr 1991; Schlupp et al. 2002). Amazon and sailfin mollies occur in sympatry from river drainages in south Texas south to drainages in north Mexico. Additionally, Amazon mollies and sailfin mollies were introduced into the San Marcos River in central Texas [*P. latipinna* introduced in the 1930s (Brown 1953); *P. formosa* introduced in the 1950s (Hubbs et al. 1953, 1991; Schlupp et al. 2002)], from which an established population has persisted.

Sailfin mollies are sexually dimorphic, with males having an enlarged dorsal fin and enhanced coloration as compared to the drab females. During courtship, the male dorsal fin is erected and presented to females and is accompanied by a sigmoid curving of the body (Travis & Woodward 1989). Males may then nibble at the gonopore of the females and may transfer sperm packets, spermatzeugmata, to the female by thrusting their gonopodium towards the females' gonopore (Constantz 1989).

Sailfin mollies are one of several species of live-bearing fish that exhibit alternative reproductive strategies in males (reviewed in Farr, 1989). Genetic polymorphism exists for body size in males, based primarily on a series of Y-linked alleles, and is associated with differences in secondary sexual characteristics and patterns of behavior (e.g., timing of sexual maturation; Farr et al. 1986; Travis & Woodward 1989). Like other poeciliids, males cease to grow after maturity (Trexler et al. 1990). There is a significant amount of variation in male body size within a population, with larger males taking up to 60 days to reach sexual maturity, while smaller males may take as few as 30 days (Travis 1994). Smaller males are more likely to attempt sneak copulations with females, as larger males are known to act aggressively toward smaller males and thereby limit the access of small males to females (Travis & Woodard 1989;

Travis et al. 1990). Additionally, females also prefer to mate, as well as to associate with larger males (Ptacek & Travis 1997; Gabor 1999; Gabor et al. 2011).

Male sailfin mollies prefer to mate with conspecifics over Amazon mollies (Ryan et al. 1996; Gabor & Ryan 2001; Gabor & Aspbury 2008; Robinson et al. 2008; Aspbury et al. 2010b; Gabor & Grober 2010), and male sailfin mollies prime more sperm for conspecifics over Amazon mollies (Aspbury & Gabor 2004b). In spite of this strong preference for conspecifics by males, Amazon mollies have persisted for ~100,000 years (Avisé et al. 1991; Scharl et al. 1995; Stöck et al. 2010). The mechanisms by which the Amazon molly has been maintained over evolutionary time have yet to be fully resolved.

Sexual Selection and Species Recognition

Sexual selection is the differential reproductive success of individuals as a result of the interplay between intersexual selection and intrasexual selection (Darwin 1871; reviewed in Andersson 1994). Generally, females are the “choosy” sex (reviewed in Andersson 1994) as the process of oogenesis is energetically more costly than spermatogenesis (Trivers 1972). However, there is an increasing amount of evidence that sperm production can be costly for males (Dewsbury 1982; Nakatsuru & Kramer 1982; Shapiro et al. 1994; reviewed in Wedell et al. 2002; Aspbury & Gabor 2004a, b). Therefore, males may exercise mate choice and can be prudent in sperm allocation (reviewed in Wedell et al. 2002).

Male mate choice is especially important in systems when closely-related heterospecifics are sympatric, as males must exercise not only mate-quality recognition but also species recognition (Pfennig 1998; Pfennig 2000; Gabor & Ryan 2001; Aspbury & Gabor 2004a, b; Gumm & Gabor 2005; Gumm et al. 2006; Robinson et al. 2008).

Individuals may be more likely to choose mates with trait values close to the population mean when emphasizing species recognition; however, individuals with exaggerated traits (i.e., trait values away from the mean) are likely to be chosen as mates when mate-quality recognition is more important, as these traits are expected to be costly to produce (Rand et al. 1992; Sherman et al. 1997; Pfennig 1998). The potential for conflict arises if heterospecifics have traits resembling high-quality conspecifics and may result in an increased frequency of heterospecific matings (Pfennig 1998). One outcome of this conflict is that individuals may use different cues to assess mate quality than those that are used to assess species identity (Pfennig 1998; Pfennig 2000), resulting in a tradeoff with individuals choosing lower-quality mates, as the risk to selecting heterospecific mate may be reduced. Recent studies have confirmed this prediction (Pfennig 2000; Candolin 2003; Gumm & Gabor 2005; Gumm et al. 2006; Gabor et al. 2010).

The unisexual-bisexual mating complex of mollies is one well-studied system in which males must discriminate between conspecifics and closely-related heterospecifics. Several studies have examined whether male sailfin mollies use multiple cues for species recognition and if they overlap with cues used for mate-quality recognition (Aspbury & Gabor 2004b; Gumm & Gabor 2005; Gumm et al. 2006; Aspbury et al. 2010b; Gabor et al. 2010). In general, these studies have found that males use a combination of female size, chemical cues and visual cues to recognize conspecifics; however, the use of size as a cue results in a conflict between mate-quality and species recognition, such that male sailfin mollies do not prefer female conspecifics over heterospecifics when the Amazon mollies are larger than the female sailfin mollies (Gumm & Gabor 2005).

Behavioral Syndromes

Recently, a framework for studying behavior has been developed which emphasizes the need to obtain a more holistic view of animal behavior (Sih et al. 2004a, b). This framework, recently coined ‘behavioral syndromes’ by Sih et al. (2004a), examines correlated traits across multiple environmental situations (Sih et al. 2004a; reviewed in Conrad et al. 2011) and is similar in principle to other terms in the literature such as “coping style” (Koolhaas et al. 1999), “personality” (Buss 1991; Gosling 2001) and “temperament” (Boissy 1995; Clarke & Boinski 1995). Studies of behavioral syndromes consist of examining whether there is a correlation between (1) multiple behaviors (i.e. ‘behavioral contexts’) in a single environmental situation, (2) a single behavior across multiple environments and/or an environmental gradient, or (3) multiple behaviors across multiple environments and/or environmental gradients (Sih et al. 2004a, b). A ‘behavioral syndrome’ is a population-level property, while a ‘behavioral type’ is an individual-level property (Bell 2007). Specifically, a ‘behavioral syndrome’ is the correlation between behavioral categories that results in a rank-order difference between individuals of a population, while a ‘behavioral type’ is the specific configuration of the behavioral syndrome that is expressed by an individual (Sih et al. 2004a,b; Bell 2007).

Numerous studies have found correlations among multiple behavioral contexts when examined in a single environmental situation (Coleman & Wilson 1998; Dugatkin & Alfieri 2003; Cote et al. 2010; Jones & Godin 2010; Smith & Blumstein 2010; Cote et al. 2011). For example, Cote et al. (2010) found that individual mosquitofish (*Gambusia affinis*) that were bold were also likely to be more active and to explore larger areas than shy individuals. Smith & Blumstein (2010) found that boldness in guppies (*Poecilia*

reticulata) served as an honest signal to predators, and these bold individuals escaped predatory attacks more often. These studies show that groups of behaviors can be correlated within a single environmental situation and that individual variation among these correlated behaviors can have important fitness consequences.

In addition to the findings that behavioral contexts can be correlated within one situation, some evidence suggests that individuals will behave consistently across different environmental situations (Gosling 2001; Sih et al. 2003; Wilson & Godin 2009). For example, a recent study by Wilson & Godin (2009) found that individual bluegill sunfish (*Lepomis macrochirus*) showed consistency in a boldness syndrome in both a novel environment and during predator inspection. Specifically, bold individuals were more likely to be active, to explore a novel environment and to inspect a predator as compared to shy individuals (Wilson & Godin 2009). Sih et al. (2003) found that boldness in larvae of the salamander, *Ambystoma barbouri*, measured as time not hiding in a refuge (i.e. exposure), was consistent across three different environments which varied in predation pressure between daytime versus nighttime exposure in pools with fish versus pools lacking fish. These studies highlight the importance of examining behaviors across multiple environmental situations because the expression of ecologically relevant behaviors (e.g., exposure to predation pressures) may appear maladaptive if studied in isolation. Furthermore, these studies suggest that across-situation conflicts, such as consistently correlated behaviors expressed across situations (Sih et al. 2003; Sih et al. 2004a,b) may explain the maintenance of sub-optimal behaviors if selection acts on the correlated traits simultaneously.

The behavioral syndrome framework is attractive because it provides a model to explain the limited plasticity of behavioral traits, termed a ‘behavioral carryover’ (Sih et al. 2004a), as well as the expression of seemingly “sub-optimal” and/or maladaptive behaviors (Sih et al. 2004a,b; Bell 2007). Older behavioral frameworks suggest that selective forces should have shaped individual behavioral responses such that individuals would express the optimal behavioral strategy in any environmental situation (reviewed in Dall et al. 2004; Dingemanse & Réale 2005). Earlier studies provided support for this hypothesis by demonstrating that individuals are able to alter levels of aggression across situations (Huntingford 1982; Riechert & Hedrick 1993), although it was found that certain individuals of a population were consistently more aggressive than others (Huntingford 1982; Riechert & Hedrick 1993; Dall et al. 2004). However, it is becoming increasingly clear that individuals may actually be constrained in their ability to tailor their behavior to their current environment (Sih et al. 2004a,b; Dingemanse & Réale 2005). These constraints can be attributed to factors such as correlational selection on multiple behaviors, which could result in selection on non-target behavioral traits (Lande & Arnold 1983; Bell 2007; Bell & Sih 2007). For example, Johnson & Sih (2005) found that female fishing spiders (*Dolomedes triton*) were more likely to engage in pre-copulatory sexual cannibalism if they had been aggressive in foraging as juveniles. This example represents a situation in which a behavioral carryover has resulted in the maintenance of a seemingly maladaptive trait, pre-copulatory sexual cannibalism, via correlational selection for juvenile aggressiveness. Limited behavioral plasticity may also arise due to physiological constraints on organisms (Bell 2007), such as pleiotropy and/or hormonal regulation of behaviors (Ketterson & Nolan 1999; Bell 2007).

Hormones and Behavior

Glucocorticoids are a class of steroid hormone found in all vertebrate species. Glucocorticoids play an important role in regulating the physiology of organisms and in the vertebrate stress response (reviewed by Sapolsky et al. 2000). Glucocorticoid release into circulation is a primary response to environmental stressors, such as chemical, physical, or perceived stressors, in most vertebrate taxa, resulting in the physiological adjustments needed for survival (Sapolsky et al. 2000). Glucocorticoids have two main classes of action: (1) ‘modulating’, which affects the response of an organism to a current stressor and (2) ‘preparative’, which allows for the organism to respond to future environmental stressors (Sapolsky et al. 2000).

Elevated levels of glucocorticoids results in a reallocation of resources from functions related to growth and reproduction to behaviors which are likely to increase immediate survival (reviewed in Wendelaar Bonga 1997; Bonier et al. 2009). In addition, empirical evidence suggests that higher levels of glucocorticoid production are correlated with a poor body condition and lower fitness of individuals (Sands & Creel 2004; Arlettaz et al. 2007; Thiel et al. 2008; Bonier et al. 2009). Therefore, levels of glucocorticoids can be used to measure the allostatic load, i.e., current and future energetic demands (McEwen & Wingfield 2003; Korte et al. 2005; Bonier et al. 2009), of an individual (or a population) and can also be used as a proxy to evaluate the health of an individual or a population (Bonier et al. 2009). These ideas lead Bonier et al. (2009) to propose the ‘Cort-Fitness Hypothesis’, which argues that there is a negative relationship between fitness and glucocorticoid production based on the premises that glucocorticoid levels should be positively correlated with increasing environmental demands and fitness

and environmental demands should be negatively correlated. However, Bonier et al. (2009) found limited support for this hypothesis and suggested that further studies are needed.

Recent evidence suggests that production of glucocorticoids could be subject to both natural and sexual selection due to the existence of variability in the stress response among individuals of a population (Husak & Moore 2008). Empirical evidence suggests that males with a worse body condition are less likely to be chosen by females as a mate than males with a better body condition (Andersson 1994). This aligns with the ‘Cort-Fitness Hypothesis’ if body condition and glucocorticoid production do indeed negatively covary, as well as if males in poor body condition are less likely to obtain matings. Although some empirical evidence suggests that short-term elevations of glucocorticoids may be adaptive (Bonier et al. 2009; Thaker et al. 2009), it is possible that sexual selection may favor individuals that consistently produce lower levels of glucocorticoids. Therefore, it is possible that production of stress hormones may influence mate choice (Husak & Moore 2008) and/or species recognition.

Production of glucocorticoids can suppress activity of the hypothalamus-pituitary-gonadal (HPG) axis, resulting in decreased secretion of gonadal hormones, thereby decreasing the expression of androgen-mediated mating behavior (reviewed by Adkins-Regan 2005). Conversely, Husak & Moore (2008) argue that one of the functions of glucocorticoid release is to ‘mobilize’ energy stores, presumably in either a ‘modulating’ or ‘preparative’ fashion (Sapolsky et al. 2000). For example, Thaker et al. (2009) found that individual male tree lizards (*Urosaurus ornatus*) with elevated glucocorticoid levels exhibited enhanced antipredator responsiveness when compared to individuals with

normal or lower levels. Therefore, it is possible that the energy stores made available via glucocorticoid release can actually facilitate reproductive behavior via an interaction with sex steroid (i.e. gonadal) hormones (Romero 2002; Moore & Jessop 2003; Husak & Moore 2008).

Cortisol is the main corticosteroid in teleost fishes (Wendelaar Bonga 1997; Mommsen et al. 1999; Arterbery et al. 2010), and has both glucocorticoid and mineralocorticoid functions in fishes (reviewed in Wendelaar Bonga 1997; Mommsen et al. 1999). Specifically, cortisol plays an important role in regulating the hydromineral balance by affecting the uptake of ions such as Na^+ and Cl^- , as well as in metabolizing carbohydrates, lipids and proteins for energy mobilization (reviewed in Wendelaar Bonga 1997). Cortisol production in fishes is an end-product of the stimulation of the hypothalamic-pituitary-interrenal (HPI) axis by either corticotropin-releasing hormone (CRH) or adrenocorticotrophic hormone (ACTH; reviewed in Wendelaar Bonga 1997). Elevated levels of cortisol can be observed relatively quickly in response to a stressor, such as handling, pollutants or environmental changes (reviewed in Wendelaar Bonga 1997).

The examination of suites of correlated traits (i.e. behavioral syndromes) can provide insight into how individuals cope with stressful situations (Koolhaas et al. 1999; Bell 2007; Thomson et al. 2011). Boldness, or an individual's propensity to take risks, is often correlated with other behaviors, such as aggressiveness, general activity and exploration tendencies (Verbeek et al. 1996; Sneddon 2003; Carere et al. 2005; van Oers et al. 2005; Magnhagen 2007; Cote et al. 2010; Smith & Blumstein 2010; Cote et al. 2011; Thomson et al. 2011). Therefore, boldness and its correlates are often associated

with either a proactive or reactive coping style (De Boer et al. 1990; Koolhaas et al. 1999), and coping style is believed to be correlated with HPI activity (De Boer et al. 1990). To my knowledge, only one study to date has examined differences in the physiological correlates of correlated traits related to boldness. This study, by Thomson et al. (2011), examined whether there was a correlation in bold behavioral types in rainbow trout, *Oncorhynchus mykiss*, which were selectively bred for higher and lower endocrine responses to stress. However, this study did not find a significant relationship between stress responsiveness via cortisol production, and behavioral type (i.e. bold vs. shy), suggesting that underlying genetic and physiological mechanisms may be inadequate to fully explain the prevalence of correlated traits and their associated behavioral phenotype.

Rationale and Objectives

I argue that further insight into the maintenance of unisexual-bisexual mating complexes might be gained by taking a more holistic view of male and female behavior. In the Amazon and sailfin molly mating complex, it is possible that the behaviors which cause males to act in a maladaptive manner (i.e. heterospecific matings) are the result of a behavioral carryover in which these same behaviors are favored, such as in conspecific mating, foraging, or predator avoidance. From a female perspective, it is possible that there will be a difference in overall syndrome composition between species, as Amazon mollies are likely to have different mate choice behavior than female sailfin mollies since Amazon mollies benefit from a higher number of matings, as compared to sailfin molly females which benefit from higher quality matings. Therefore, in this thesis, I examine some proximate mechanisms which may underlie the presence of behavioral syndromes,

as well as the ultimate consequences of behavioral syndromes in explaining the maintenance of this unisexual-bisexual mating complex of mollies.

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CHAPTER II

INDIVIDUAL VARIATION IN MALE BEHAVIORAL TYPE AND ITS CORRELATES WITH MALE MATE PREFERENCE AND STRESS HORMONES

Abstract

The persistence of gynogenetic organisms is an evolutionary paradox. An ideal system for examining the persistence of gynogens is the unisexual-bisexual mating complex of the unisexual Amazon molly (*Poecilia formosa*), and the bisexual, host species, the sailfin molly (*P. latipinna*) and the Atlantic molly (*P. mexicana*). Insight into the maintenance of this mating complex might be enhanced by taking a more holistic view of male and female behavior through a behavioral syndrome framework. In this study, we examined whether behavioral syndromes are correlated with male mate-choice and stress hormone production. We quantified behaviors related to activity, boldness, exploration and sociability in male sailfin mollies, as well as their mate preference for conspecific females or the all-female species of Amazon mollies. In addition, we examined baseline cortisol production in male sailfin mollies. The results of this study provide evidence for behavioral syndromes in male sailfin mollies, but individual behavioral type was not correlated with their mate preference or stress hormone production. However, we did find differences in baseline cortisol production related to male boldness behaviors. The lack of

correlation between behavioral syndromes, mate preference and stress hormone production emphasizes that the nature of behavioral-hormonal interactions are complex. In summary, neither individual traits nor the behavioral syndromes found here are adequate to explain the maintenance of this unisexual-bisexual mating system.

Introduction

The persistence of gynogenetic organisms is an evolutionary paradox. Gynogenetic organisms reproduce asexually and require sperm from closely related heterospecifics to induce egg development; however, paternal genetic information is not incorporated into the genome of the offspring (Kallman 1962; Schultz 1967, but see Bogart et al. 1989). As such, gynogens are often considered sexual parasites of their host species (Hubbs 1964), as males obtain no known benefits from mating with these females (but see Schlupp et al. 1994). In addition, gynogenetic organisms are disadvantaged as they are unable to purge deleterious mutations due to the lack of recombination (Muller 1964). These factors suggest that gynogenetic lineages should be short-lived, but several vertebrate, gynogenetic lineages have been maintained over time.

An ideal system for examining the persistence of gynogens is the unisexual-bisexual mating complex consisting of the bisexual, parent species, the sailfin molly (*Poecilia latipinna*) and the Atlantic molly (*P. mexicana*), and the unisexual, gynogenetic Amazon molly (*P. formosa*). Amazon mollies are a clonal, all-female species of live-bearing fish of hybrid origin (Hubbs & Hubbs 1932; Dawley 1989). Sperm from one of the parent species is required by the Amazon molly to start embryogenesis (Hubbs & Hubbs 1932).

In general, male sailfin mollies prefer to mate with conspecifics over Amazon mollies (Ryan et al. 1996; Gabor & Ryan 2001; Gabor & Aspbury 2008; Robinson et al. 2008; Aspbury et al. 2010; Gabor & Grober 2010), and male sailfin mollies prime more sperm for conspecifics over Amazon mollies (Aspbury & Gabor 2004 a,b). In spite of this strong preference for conspecifics by male sailfin mollies, Amazon mollies have persisted for ~100,000 years (Avice et al. 1991; Schartl et al. 1995; Stöck et al. 2010). The mechanisms underlying the maintenance of Amazon mollies have yet to be fully understood.

Insight into the maintenance of unisexual-bisexual mating complexes might be enhanced by taking a more holistic view of male and female behavior through a behavioral syndrome framework (Sih et al. 2004a,b). In general, studies of behavioral syndromes examine correlations among multiple behaviors at the population level, as well as the consistency in the behavior of individuals across environmental situations (Sih et al. 2004a, b; Bell 2007). The behavioral syndrome framework provides a model to explain observations of limited plasticity in behavioral traits, termed a ‘behavioral carryover’ (Sih et al. 2004a), which may result in the expression and maintenance of seemingly “sub-optimal” and/or maladaptive behaviors (Sih et al. 2004a,b; Bell 2007). It is becoming increasingly clear that individuals may be constrained in their ability to tailor behavior to their current environment (Sih et al. 2004a,b; Dingemanse & Réale 2005). These constraints can be attributed to factors such as correlational selection on multiple behaviors, which could result in indirect selection on non-target behavioral traits (Lande & Arnold 1983; Bell 2007; Bell & Sih 2007). For example, Johnson & Sih (2005) found that female fishing spiders (*Dolomedes triton*) were more likely to engage in pre-

copulatory sexual cannibalism if they foraged aggressively as juveniles. This behavioral carryover has resulted in the maintenance of a seemingly maladaptive trait, pre-copulatory sexual cannibalism, via correlational selection for juvenile aggressiveness. Limited behavioral plasticity may also arise due to physiological constraints on organisms (Bell 2007), such as pleiotropy and/or hormonal regulation of behaviors (Ketterson & Nolan 1999; Bell 2007).

Circulating hormones regulate behaviors in a variety of ways. Gabor & Grober (2010) found that 11-ketotestosterone (KT), the main fish androgen, may influence male mate preference and species recognition abilities of sailfin mollies, as sailfin mollies of both sexes exhibited an increase in KT release rates when mating together; however, no such increase in KT was observed when males mated with Amazon mollies. Elevated levels of glucocorticoids (stress hormones) can result in a reallocation of resources from functions related to growth and reproduction to behaviors that are likely to increase immediate survival (reviewed in Wendelaar Bonga 1997; Milla et al. 2009; Schreck 2010). Cortisol is the main glucocorticoid in teleost fishes (Wendelaar Bonga 1997; Mommsen et al. 1999; Arterbery et al. 2010), and cortisol production in fishes is an end-product of the stimulation of the hypothalamic-pituitary-interrenal (HPI) axis by either corticotropin-releasing hormone (CRH) or adrenocorticotrophic hormone (ACTH; reviewed in Wendelaar Bonga 1997). Because hormones regulate behavior, it is useful to examine the relationship between hormones and correlated behaviors (i.e., behavioral syndromes).

Boldness, an individual's propensity to take risks, and its correlates (e.g. activity, aggression, and exploration) are associated with how individuals react to stressful

situations (De Boer et al. 1990; Koolhaas et al. 1999), and this reaction may be correlated with HPI activity in fishes (De Boer et al. 1990). A recent study by Archard et al. (2012) discovered a significant, positive relationship between cortisol production and measures of exploratory tendencies and activity in wild-caught *Brachyrhaphis episcopi*.

Exploration and activity traits are known correlates to boldness (Cote et al. 2010; Smith & Blumstein 2010; Cote et al. 2011). However, Thomson et al. (2011) examined the correlation between bold behavioral types in rainbow trout, *Oncorhynchus mykiss*, which were selectively bred for higher and lower endocrine responses to stress, but they did not find a significant relationship between stress responsiveness and behavioral type (i.e., bold vs. shy). These studies highlight the complex nature of hormone-behavior interactions and suggest that natural and artificial selection may have different effects on the expression of this interaction.

In the Amazon and sailfin molly mating complex, it is possible that a behavioral carryover could explain the maintenance of the maladaptive behavior in males of mating with a heterospecific female. For example, prior studies have shown that bold males are more likely to inspect a predator (Godin & Dugatkin 1996; Smith & Blumstein 2010) and that bold males were more likely to obtain mates (Godin & Dugatkin 1996). Therefore, we hypothesize that there will be a relationship between boldness (and its correlates) and male sailfin molly mating preference in the unisexual-bisexual system. We predict that males with a more active/bold/exploratory/social behavioral type will have a higher strength of preference (SOP) for conspecifics as a result of increased inspection time with females. In addition, we hypothesize that there is a relationship between stress hormones and correlated behaviors. While there has been mixed support for this hypothesis

(Thomson et al. 2011; Archard et al. 2012), we predict that individuals with an active/bold/exploratory/social behavioral type will have higher baseline levels of cortisol, as these behaviors could allow individuals to cope with stressful situations and return to normal activities more quickly.

Methods

Fish Maintenance

Male and female sailfin mollies, as well as Amazon mollies, were collected from the headwaters of San Marcos River, Spring Lake, San Marcos, Hays County, TX (29.89 N, 97.82 W) from April-September 2011. Fishes were maintained in the laboratory for at least two weeks prior to experimentation. Males were maintained in mixed-sex aquaria (38-L). All fish were kept on a 14:10 L:D cycle and were fed a combination of brine shrimp (*Artemia* spp.; Bio-Marine; www.aquafauna.com) and Spirulina Flakes mixed with Aquamax[®] Fry Starter 200 (PMI Nutrition International) once daily between 1630 - 1730 h to control for any hormonal changes due to feeding.

Identification of Behavioral Types

To examine behavioral types, individual males (n=50) were exposed to each of four treatments to assess: (1) activity/boldness/exploration, (2) sociability, (3) male mate choice, and (4) repeatability of activity/boldness /exploration (repeat of treatments 1 & 2). All tests occurred between 0800-1700h, and treatments 3 & 4 occurred on the days following treatments 1 & 2. The first two treatments were executed to identify correlations among different behavioral metrics, while treatment 3 examined male mate preference, and treatment 4 examined the repeatability and consistency of individual behavior over time.

Treatment 1: Activity/Boldness/Exploration Assay + Hormone Assay

Treatment 1 quantified activity, boldness and exploration in a novel environment using methods adapted from Cote et al. (2010) and Smith & Blumstein (2010). First, we placed individual males into an opaque, acclimation chamber (15cm in diameter) for 5 min within an experimental arena (70x40x10 cm), which was marked with a grid of 36 total squares (each 7.5x8.5 cm). After acclimation, we remotely opened a door located on the exterior of the chamber and then recorded the time to emerge for up to 10 min. We quantified *boldness* as (maximum time to exit) - (individual time to exit), such that individuals that exited more quickly had higher scores than those that exited more slowly. We also measured time spent swimming (*activity 1a*) in the experimental arena, as well as the number of grids entered (*exploration*) for 5 min after emergence from video following the trial.

We obtained baseline cortisol release rates via water-borne hormone samples levels 24h before Treatment 1. Hormone samples were taken between 0800-1300h to control for circadian variation in hormone levels (Lorenzi et al. 2008). Water-borne hormone collection methods followed Gabor & Grober (2010). Briefly, individual males were placed into a 250 mL beaker filled with 100 mL of conditioned water and then removed after 1 hour. Water samples were stored at -20 °C until hormone assays were completed at a later date (Ellis et al. 2004). All subsequent hormone assay procedures followed the methods of Gabor & Grober (2010). To assay cortisol levels, we used commercially available enzyme-immunoassay (EIA) kits (Cayman Chemical). Gabor & Contreras (in review) validated the use of water-borne hormones to measure cortisol on EIA plates, as well as the correlation between water-borne release rates and plasma

cortisol production for sailfin mollies. All samples were run in duplicate on 96-well plates and read by a fluorescent plate reader (BioTek Powerwave XS).

Treatment 2: Sociability Assay

This treatment examined individual sociability using methods adapted from Cote et al. (2010) and Smith & Blumstein (2010) and took place in a 75-L dichotomous-choice tank, which was divided into a large, center compartment (60cm; *no-choice zone*) and two smaller compartments (each 10 cm) with sealed, plexiglass dividers. The *preference zone* was defined as the 10 cm in front of each small side compartment. One side compartment housed a mixed-species shoal during testing, while the other (control) contained only water. Shoals contained two female sailfin mollies and two Amazon mollies, and we randomized shoal placement (i.e., right vs. left) for each focal individual. Females were housed together in 38-L, mixed-species aquaria prior to testing, but were divided into artificial shoals on the day of testing. Finally, we randomized which one of the two shoals was presented to the males. First, we habituated the focal male for 5 min under an opaque, acclimation chamber (16cm in diameter) in the center of the no-choice zone. After acclimation, we removed the chamber and recorded the time spent in the preference zone (*sociability*), as well as total time swimming (*activity 2a*) for 10 min.

Treatment 3: Male Mate-Choice

This treatment examined the mate preference of male sailfin mollies. One sailfin molly male was housed with a size-matched female pair, consisting of one female sailfin molly and one Amazon molly (female standard length (SL) within ± 2 mm of each other and within ± 9 mm male SL). Groups of fish were housed for 18-24 hours in individual aquaria (19-L), with a transparent divider separating the sexes. Females were isolated

from males either for 30 days or, if housed individually, were used approximately one week after parturition of fry.

On the day following Treatments 1 and 2, males were given access to the female pair. The central divider was removed, and the time until the first gonopodial thrust (i.e., mating attempts) toward a female was recorded. Following the first thrust, the total number of thrusts by the male at each female was recorded for 10 min. At the end of the trial, females were removed from the aquaria. If no mating behavior was observed, the trial ended after 30 min. We measured strength of preference (SOP) as the number of thrusts towards conspecifics/total number of thrusts.

Treatment 4: Repeatability of Behavior

This treatment examined the repeatability of behaviors measured in Treatment 1. Repeatability is the intraclass correlation coefficient based on variance components obtained from a one-way Analysis of Variance (ANOVA) and can represent the upper-limits on the heritability of a trait (Lessels & Boag 1987). Measures of repeatability are necessary in studies of behavioral syndromes to examine individual changes in trait (i.e., behavioral) expression across time relative to changes at the population level (Lessels & Boag 1987; Bell et al. 2009; Cote et al. 2010). The behavioral syndrome framework predicts that individuals should maintain a consistent pattern of behavior over time; however, most studies find relatively low levels of repeatability (Bell et al. 2009; Cote et al. 2010). Additionally, we examined the consistency of behaviors by conducting a Spearman's rank correlation to compare the change in individual ranks for each behavioral metric over a two-day time span.

Statistical Analyses

Data from all treatments could not be transformed to meet the assumptions of parametric analyses; therefore, we used non-parametric analyses. To identify behavioral syndromes, we first examined whether there were significant, pair-wise correlations among the behavioral metrics with Spearman's rank correlation. Fish size (standard length: SL) was not significantly correlated with any behavioral metric and was not included in further analyses (Table 1). Since the behavioral metrics were all significantly correlated (except for sociability-exploration; Table 1), we used a Principal Components Analysis (PCA) to identify multiple correlations among behaviors (i.e., personality-trait dimensions; Cote et al. 2010). The use of PCA allowed us to extract component scores from the first two principal components (Table 2). Behavioral metrics with a loading of 0.32 or greater were considered to be strongly associated with that principal component (Tabachnick & Fidell 1996). Component scores from PCA are analogous to individual behavioral type; therefore, the results from PCA are necessary to examine the relationship between behavioral type and male mate preference, as well as between behavioral type and stress hormone production, in this study.

We used Spearman's rank correlation to examine whether there was a relationship between the individual behavioral metrics and SOP, as well as between individual component scores (i.e., PC1 & PC2 scores) and SOP. We also used Spearman's rank correlation to examine the relationship between baseline cortisol production and the individual behavioral metrics, as well as the individual component scores obtained from the PCA. We used a Wilcoxon signed ranks test to compare differences in number of gonopodial thrusts and latency to thrust at conspecific versus heterospecific females.

Additionally, we used a Mann-Whitney U-test to examine differences in baseline cortisol release rates between males that exited the acclimation chamber in Treatment 1 and those that did not.

Results

Identification of Behavioral Syndromes

There were significant, pair-wise correlations among all behavioral metrics (except for sociability-exploration; Table 1), which indicates the presence of a behavioral syndrome in this population of male sailfin mollies. The measured behaviors loaded onto two, orthogonal principal components that explained approximately 83% of the variance in the expression of behavior among individual males (Table 2). Based on the component loadings, principal component 1 (PC1) is positively associated with activity measures from treatments 1 and 2 [i.e. ‘Activity (1a)’ and ‘Activity (2a)’], as well as with boldness, exploration, and sociability (Table 2). Principal component 2 (PC2) is positively associated with activity in treatment 2 [i.e. Activity (2a)] and with sociability, and is negatively associated with activity in treatment 1 [i.e. Activity (1a)] and exploration (Table 2). Therefore, individuals with positive PC1 scores can be characterized as being more active, bold, exploratory and social than individuals with negative PC1 scores. Individuals with positive PC2 scores can be characterized as active and social but shy and non-exploratory, whereas the opposite is true for individuals with negative PC2 scores).

Repeatability of Behaviors

Individual males were highly consistent and repeatable in their boldness, exploratory and activity behavior (Table 3).

Behavioral Syndromes and Mate Choice

Males preferred to mate with conspecific versus heterospecific females (Wilcoxon signed ranks: $z=3.95$, $p<0.0001$; Figure 1). Male strength of preference (SOP) for conspecifics was not significantly correlated with any of the individual, behavioral metrics (Table 1). Additionally, SOP was not significantly correlated with individual component scores for PC1 ($\rho = -0.0496$, $p=0.744$) nor with individual component scores for PC2 ($\rho=0.0061$, $p=0.968$). There was a trend toward significance in the correlation between PC2 scores and the latency to thrust at a conspecific female ($\rho = -0.2648$, $p=0.063$), as well as between PC2 scores and the number of thrusts towards heterospecific females ($\rho=0.2685$, $p=0.059$). This suggests that more social individuals took less time to thrust towards a conspecific female, but thrusted more at heterospecific females, than less social males.

Behavioral Syndromes and Stress Hormones

There was not a significant correlation between baseline cortisol release rates and the behaviors examined in Treatment 1 or individual component scores for PC1 (Table 4). In this experiment, 15 of the 50 males did not exit the acclimation chamber, and there was a significant difference in baseline cortisol release rates between those individuals that did and those that did not exit the acclimation chamber (Mann-Whitney: $U = -2.30$, $df=3$, $p=0.02$). Males that did exit the acclimation chamber had significantly higher baseline levels of cortisol production than males that did not exit (Figure 2).

Discussion

Evidence for Behavioral Syndromes

We found evidence for the presence of behavioral syndromes in this population of male sailfin mollies. We found significant, pair-wise correlations among all the behavioral metrics (except for sociability-exploration; Table 1). The presence of behavioral syndromes is also supported by the multiple correlations among behaviors that loaded onto two, orthogonal principal components (Table 2). Additionally, individual males were highly consistent and repeatable in their behavior over two days (Table 3). The values for repeatability found here (Table 3) appear to be average-high in strength when compared to the findings of a meta-analysis conducted by Bell et al. (2009).

To date, two other studies have examined the role that behavioral syndromes play in shaping behavior in the unisexual-bisexual mating complex of mollies. Scharnweber et al. (2011) examined boldness traits in female sailfin and Amazon mollies and found that the expression of boldness traits was similar across species. However, they were unable to detect an overall behavioral syndrome due to lack of correlations between the measured behavioral traits, possibly due to small sample sizes. In a second study that used male sailfin mollies from a population allopatric to Amazon mollies, Seda et al. (2012) found a relationship between courtship and boldness traits and determined that certain combinations of behaviors within the behavioral syndrome were size-dependent while others were not associated with male size.

We found multiple correlations among activity (1a), boldness and exploratory behaviors and activity (2a) and sociability behaviors (Table 2). These results are congruent to the findings of Cote et al. (2010), as they found the same correlations among

behaviors, except that they did not measure activity in their second treatment. Our results differ from those of Scharnweber et al. (2011) in that we found evidence of correlated behavioral traits. Additionally, our results somewhat differ from those of Seda et al. (2012) in that we did not find a significant correlation between mating behaviors (i.e. SOP) and boldness, nor did we find significant correlations between male size and the measured behaviors (Table 1).

Behavioral Syndromes and Mate Preference

In this study, we examined whether male sailfin mollies preferred conspecific females to heterospecifics and whether there was a relationship between individual behavioral type and their mating preference. We found that males preferred to mate with female sailfin mollies over Amazon mollies (Figure 1). This conspecific mating preference is similar to the findings of previous studies (Ryan et al. 1996; Gabor & Ryan 2001; Gabor & Aspbury 2008; Robinson et al. 2008; Aspbury et al. 2010; Gabor & Grober 2010). However, we did not detect a clear relationship between individual behavioral type and male mate preference. For instance, male strength of preference for conspecifics (SOP) was not correlated with individual behavioral metrics (Table 1) or composite behavioral metrics (PC1 & PC2 scores).

There was some evidence that activity (2a) and social behaviors may influence mating activity, as individual scores along the PC2 axis showed a trend toward a negative correlation with latency to thrust at conspecific females and a positive correlation with total number of thrusts directed at heterospecific females. These trends suggest that more social males took less time to thrust at conspecific females but were more likely to exhibit gonopodial thrusts towards heterospecific females than asocial males. This result

is similar to the findings of Seda et al. (2012) that found that sociability, exploration and gonopodial thrust rate towards females loaded significantly onto one principal component axis, although they also did not find a significant, pair-wise correlation between sociability and gonopodial thrust rate.

Even though we found multiple correlations among activity, boldness and exploratory behaviors, which could be the end-product of selection on correlated traits, it does not appear as if these correlated behaviors influence the mate preference of male sailfin mollies. Instead, the results presented here, and those of Seda et al. (2012), may suggest that social behaviors play a greater role in shaping male sailfin molly mating decisions. Future studies should examine the relationship between other social contexts (e.g. willingness to shoal, dispersal tendencies, etc) and male mate preference.

In this study, we predicted that males with a more active/bold/exploratory/social behavioral type would have a higher strength of preference for conspecifics as a result of increased inspection time with females. While we did not find support for this prediction, this could be an important direction to undertake in future research. Sailfin mollies are one of several species of live-bearing fish which exhibit alternative reproductive strategies in males (reviewed in Farr 1989), such that smaller males are more likely to attempt sneak copulations with females, as larger males are known to act aggressively toward smaller males and thereby limit the access of small males to females (Travis & Woodard 1989; Travis et al. 1990). Additionally, models by Kawecki (1988) and Schmeller et al. (2005) predict that as inspection time increases, the probability of mating with a heterospecific female decreases. Given that smaller male sailfin mollies are more likely to sneak copulate (Travis & Woodard 1989; Travis et al. 1990), it is possible that

smaller males will mate with a heterospecific as a result of decreased inspection time of females. Therefore, it would be interesting to examine whether there is a consistent behavioral type across dominant and subordinate males and how this would affect male mating decisions.

Behavioral Syndromes and Hormones

In this study, we also examined whether individual, male behavioral type was correlated with baseline stress hormone (cortisol) release rates. We did not find any significant correlations between baseline measures of cortisol release rates and activity, boldness or exploratory behaviors (Table 4). This result corroborates the findings of Thomson et al. (2011), who did not find a relationship between cortisol production and boldness in rainbow trout, *Oncorhynchus mykiss*. Individual differences in correlated behaviors may be too complex to be explained by single physiological or genetic factors.

We did find evidence that the baseline cortisol release rates were higher in fish that did exit the acclimation chamber in Treatment 1 compared to those that did not (~30% of tested males; Figure 2). In this situation, males that did not exit would be considered shy, while males that did exit would be considered bold. This result supported our prediction that individuals with a bold/active/exploratory behavioral type would release more cortisol compared to individuals with a shy/inactive/non-exploratory/asocial behavioral type. A possible explanation for this result is that high cortisol release rates may be associated with the behavioral type in which individuals cope with stressful events more effectively (i.e. return to normal activities more quickly). For example, a study by Thaker et al. (2009) found that elevated levels of glucocorticoids can result in increased survival of individual tree lizards (*Urosaurus ornatus*) via enhanced anti-

predator responsiveness. Future studies could examine whether there is consistency in the relationship between stress hormones and boldness across different contexts, such as in predator inspection or novel object inspection.

To date, several studies have linked boldness traits with other traits such as exploration tendencies and activity (Cote et al. 2010; Smith & Blumstein 2010; Cote et al. 2011), and these traits may be linked with organismal stress responses (De Boer et al. 1990; Koolhaas et al. 1999). We did not find a relationship between the correlated behavioral traits and cortisol release rates in our system, whereas a recent study by Archard et al. (2012) linked active and exploratory traits to cortisol release rates. One important distinction between our study and the latter is that the cortisol response was apparent after individuals from sites differing in population pressure were subjected to a mild stressor. This highlights the effects that differential selective pressures across populations can have on regulating the physiological and behavioral profiles of individuals (Archard et al. 2012). Future studies should examine the configuration of correlated behavioral traits and their relationship with stress hormone production in different populations of sailfin mollies. Specifically, it would be interesting to examine this effect across populations that differ in predation pressure, as well as across populations that are either allopatric to or sympatric with the unisexual Amazon molly.

Production of glucocorticoids can suppress activity of the hypothalamus-pituitary-gonadal (HPG) axis, resulting in decreased secretion of gonadal hormones, thereby decreasing the expression of androgen-mediated mating behavior (reviewed by Adkins-Regan 2005; Milla et al. 2009). Additionally, Gabor & Grober (2010) showed that KT plays a role in the species recognition abilities of sailfin molly males. Subsequently,

Gabor & Contreras (in review) found no correlation between baseline cortisol release rates and baseline KT release rates for these males and cortisol release rates were not significantly correlated with the number of times males attempted to mate. These results indicate that baseline cortisol release rates are not suppressing male mating behaviors in this system.

Conclusions

Taken together, these results suggest that the individual behaviors measured here, as well as the composite behavioral type of the individual males, were inadequate to fully explain the mate preference of male sailfin mollies. Activity, boldness and exploration traits are not correlated with male mate preference or species recognition abilities in this system. This result did not support our predictions, or the results of previous studies that have found that bold individuals were more likely to obtain a mating than shy individuals (Godin & Dugatkin 1996). One hypothesis is that we need to examine other traits such as attentiveness, cognitive abilities or permissiveness to fully understand the basis for male mating decisions.

Moreover, there was no significant correlation between baseline cortisol release rates and the behaviors examined or individual component scores for PC1 (Table 4). While we did provide some evidence for a relationship between boldness and baseline stress hormone release rates, our results suggest that the presence of correlated traits are not explained by individual, physiological factors. However, it would be interesting to experimentally determine the role that selective forces play in shaping the behavior-hormone interaction both within and among populations.

In sum, these results suggest that there is not the predicted relationship between boldness (and its correlates) and male sailfin molly mating preference in this unisexual-bisexual system. This indicates that while suites of correlated traits may explain the maintenance of maladaptive behaviors across some situations, they do not in the mating context of this unique mating system. Additionally, we have provided some evidence of a relationship between boldness and stress hormones, though this relationship is neither the driving force behind the correlated traits nor the expression of mating activity of males in this system.

Acknowledgements

We thank A. Sih for his insightful contributions to this project. Additionally, we thank G. Aron, F. Horne, R. Mclean, and C. Nice for loaning space and equipment needed in this study. We also thank the EEB discussion group at Texas State University-San Marcos for helpful comments on the manuscript. Finally, we thank members of the GASP lab, especially L. Alberici da Barbiano, for their help with catching and maintaining the fish. This work was funded by a National Science Foundation Grant, as well as a Grant-in-Aid of Research from Sigma Xi (to JJM). Fishes were collected in Texas under scientific collection permit no. SPR-0604-389. Our experimental protocol was reviewed and approved by Texas State University Institutional Animal Care and Use Committee (protocol 1032_0924_26).

Tables

Table 1. Spearman's rank correlations among the five behavioral categories that were measured, also including standard length (SL) and male strength of preference for conspecifics (SOP).

	Activity 1a	Activity 2a	Boldness	Exploration	SL	Sociability
Activity 2a	0.434**	--	--	--	--	--
Boldness	0.690***	0.385**	--	--	--	--
Exploration	0.932***	0.407**	0.653***	--	--	--
SL	-0.052	-0.099	0.0002	-0.030	--	--
Sociability	0.294*	0.486**	0.362**	0.250 ^a	-0.031	--
SOP	-0.078	-0.070	0.090	-0.069	-0.012	-0.012

*p<0.05, **p<0.01, ***p<0.0001

^ap=0.08

Table 2. Component loadings of behaviors onto two, orthogonal principal components and the variance explained by each. Behavioral metrics with loadings of 0.32 or greater are considered to be associated with a principal component.

Behavior	PC 1	PC 2
Activity 1a	0.492	-0.367
Activity 2a	0.413	0.561
Boldness	0.416	-0.268
Exploration	0.508	-0.348
Sociability	0.395	0.599
Variance explained (%)	59.001	23.585
Cumulative Variance Explained (%)	59.001	82.585

Tables (continued)**Table 3.** Individual consistency (Spearman's rank correlation) and repeatability (intraclass correlation coefficient; ICC) of the three behaviors measured in Treatment 1 over two days.

Behavioral Context	Rank Correlation (ρ)	Repeatability ICC \pm SE
Activity (1)	0.56 p<0.0001	0.47 \pm 0.16 F _{49,99} =2.77, p<0.0001
Boldness	0.49 p=0.0003	0.40 \pm 0.17 F _{49,99} =2.35, p=0.002
Exploration	0.59 p<0.0001	0.51 \pm 0.15 F _{49,99} =3.04, p<0.0001

Table 4. Spearman's rank correlations between the behavioral contexts associated with Treatment 1, as well as individual component scores (PC1), and baseline stress hormone release rates.

	Baseline Cortisol (pg/SL/hour)	p-value
Activity 1a	0.2154	0.2211
Boldness	0.3246	0.0611
Exploration	0.2706	0.1217
PC 1	0.2196	0.2122

Figures

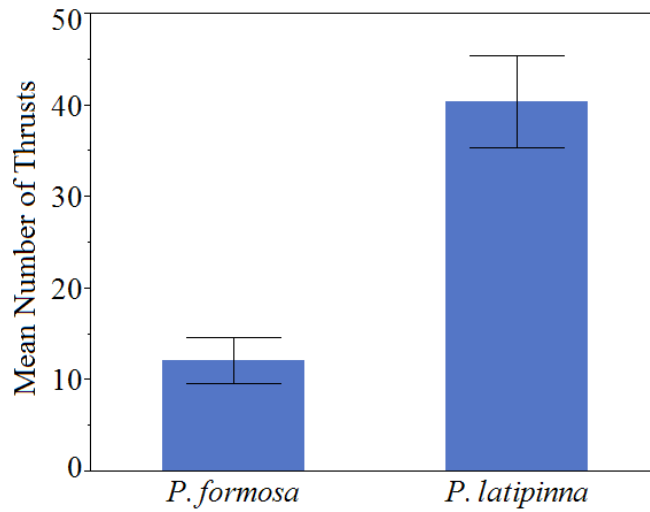


Figure 1. Number of gonopodial thrusts (\pm SE) by male sailfin mollies ($n=50$) to heterospecific (*P. formosa*) and conspecific (*P. latipinna*) females.

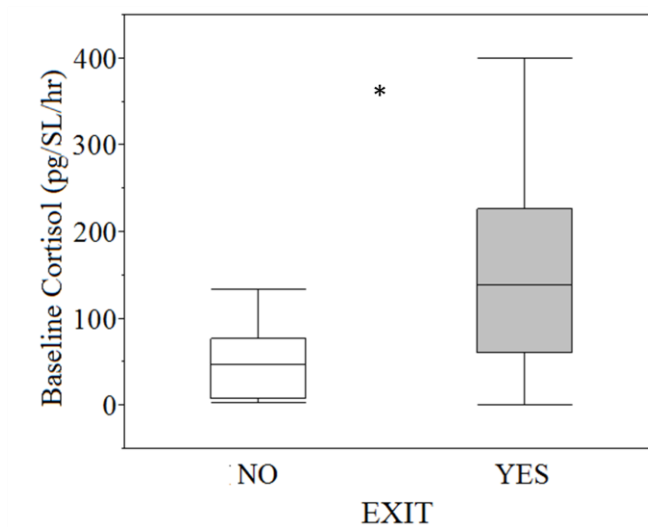


Figure 2. Box plot representing baseline cortisol release rates ($\text{pg SL}^{-1} \text{ hour}^{-1}$; median \pm 1st and 3rd quartiles) in males which did (YES; gray) or did not (NO; white) exit the acclimation chamber in Treatment 1. * indicates $p < 0.05$.

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CHAPTER III

EXAMINATION OF CORRELATED BEHAVIORAL TRAITS AND STRESS HORMONE PRODUCTION BETWEEN TWO SPECIES IN A UNISEXUAL-BISEXUAL MATING COMPLEX

Abstract

In recent years, there has been a surge of research that has examined the ecological and evolutionary implications of suites of correlated behavioral traits (i.e. behavioral syndromes). Populations have been found to differ in their behavioral syndrome expression, generally attributed to differences in selective pressures (e.g., predation). However, differences in behavioral syndromes between closely-related species that occur in sympatry have rarely been examined. These studies allow for examining differences in behavioral syndromes in terms of differences (or similarities) in genetic variation between species. We examined differences in behavioral syndromes in a mating complex of mollies, consisting of the unisexual Amazon molly (*Poecilia formosa*) and the bisexual, parent species, the sailfin molly (*P. latipinna*) and the Atlantic molly (*P. mexicana*). When female sailfin mollies and Amazon mollies occur in sympatry, there is direct competition for matings which could affect the behavioral syndromes found in each species. We examined whether Amazon mollies and female sailfin

mollies differ in behavioral syndrome composition. Additionally, we examined whether there was a relationship between stress hormone production and individual, behavioral type, as well as if there was a difference in stress hormone production between species. We identified behavioral syndromes in both species, which differed between species. We found evidence for a relationship between baseline stress hormones, the stress response and boldness in female sailfin mollies. Furthermore, we found significant differences in baseline stress hormone production and the stress response between species. The results of this study provide both a proximate and ultimate explanation to the exhibition of behavioral syndrome differences between species in this unisexual-bisexual mating complex.

Introduction

Recently, a framework for studying behavior has been developed which emphasizes the need to obtain a more holistic view of animal behavior through the examination of suites of correlated traits across multiple environmental situations (Sih et al. 2004a,b; reviewed in Conrad et al. 2011). This framework has been termed ‘behavioral syndromes’ and is similar in principle to other terms in the literature such as “coping style” (Koolhaas et al. 1999) and “personality” (Buss 1991; Gosling 2001). The behavioral syndrome framework recognizes that there is often consistent, individual variation in behavior both within and across environmental situations, which can have important ecological and evolutionary consequences (Gosling 2001; Sih et al. 2004a; Dingemanse & Réale 2005; Bell et al. 2009). For instance, the ‘constraint hypothesis’ suggests that traits become correlated through proximate mechanisms, e.g. pleiotropy or hormonal effects on multiple targets, such that selection on one trait results in selection

on the correlated trait(s) as a byproduct (Lande & Arnold 1983; Ketterson & Nolan 1999; Bell 2005; Bell 2007). Therefore, groups of correlated traits may act as a constraint on the independent evolution of individual traits. Additionally, correlational selection on non-target behavioral traits may explain the maintenance of maladaptive behaviors within a population (Lande & Arnold 1983; Bell 2007; Bell & Sih 2007),

An alternative to the ‘constraint hypothesis’, termed the ‘adaptive hypothesis’, is that correlated traits are adaptations present within populations as a result of selection on the correlation itself, resulting in the loss of maladaptive traits (Cheverud 1996; Wilson 1998; Bell 2005). Given that a behavioral syndrome is a population-level property (Bell 2007), the latter hypothesis allows researchers to make predictions about the presence and maintenance of behavioral syndromes between populations and even between species (Bell 2005). Specifically, the ‘constraint hypothesis’ predicts that behavioral syndromes should be similar across populations because of the underlying, proximate mechanisms. Conversely, the ‘adaptive hypothesis’ suggests that correlated behaviors (or perhaps the lack thereof) should differ between populations, as selection regimes will vary among geographic regions (Bell 2005). There has been more support for the ‘adaptive hypothesis’, as several studies have found differences in behavioral syndromes across populations (Bell 2005; Brown et al. 2005; Brown et al. 2007b; Dingemanse et al. 2007). The most common finding is that individuals from high predation-risk populations are often more bold (i.e. they tend to engage in risk-taking behaviors) than individuals from populations where the risk of predation is low (Bell 2005; Brown et al. 2005; Brown et al. 2007b). Furthermore, Dingemanse et al. (2007) found that three-spined sticklebacks (*Gasterosteus aculeatus*) from high-predation populations exhibited behavioral

syndromes, whereas populations in low-predation areas had only weak (or no) correlations among behaviors. Taken together, these results falsify the ‘constraint hypothesis’, suggesting that predation is one of the selective forces that can generate and maintain behavioral syndromes, while allowing for differences between populations (Bell & Sih 2007).

Given that selective forces can generate differences in suites of correlated traits between populations, it is likely that these forces will generate differences in the proximate mechanisms that affect behavior between populations as well (i.e., the opposite of the ‘constraint hypothesis’). Specifically, and as predicted by the ‘adaptive hypothesis’, the proximate mechanisms behind behavior should be locally adapted, and therefore should differ between populations with differing selective regimes (Wingfield 2005; Romero et al. 2009; Archard et al. 2012). Furthermore, as behavioral syndromes predict consistent, individual variation within a population, it is possible that there will be differences in the proximate mechanisms between individuals of a population that differ in their specific configuration of the behavioral syndrome (i.e. their ‘behavioral type’).

In recent years, several studies have examined the physiological mechanisms underlying consistent, individual differences in behavior, and most studies have examined the correlation between the stress response in individuals and their associated behavioral type (see Carere et al. 2010 for a review). The stress response results in elevated levels of glucocorticoids (stress hormones), which can result in the reallocation of resources from functions related to growth and reproduction to behaviors which are likely to increase immediate survival (reviewed in Wendelaar Bonga 1997; Milla et al. 2009; Schreck 2010). However, there has been mixed support for a relationship between

stress hormones and behavioral type in non-avian species (see Cockrem 2007 for avian examples). For example, a recent study by Thomson et al. (2011) did not detect a relationship between the individual stress response, as measured by cortisol production, and behavioral type (e.g. bold vs. shy individuals) in rainbow trout, *Oncorhynchus mykiss*. Conversely, Archard et al. (2012) discovered a significant, positive relationship between cortisol production and measures of exploratory tendencies and activity, known correlates to boldness (Cote et al. 2010; Smith & Blumstein 2010; Cote et al. 2011), in wild-caught Panamanian bishops, *Brachyrhaphis episcopi*. Additionally, Muraco et al. (*in prep.*) found that bold individuals had higher baseline release rates of cortisol than shy individuals in male sailfin mollies (*Poecilia latipinna*).

Numerous studies have examined differences in behavioral syndrome composition between populations (e.g. Bell 2005; Brown et al. 2005; Brown et al. 2007b; Dingemanse et al. 2007); but an overlooked angle is that of how syntopic species differ in behavioral syndromes. While studies of this nature cannot explicitly test the constraint or adaptive hypotheses, they do allow for predictions to be made about similarities (or differences) in behavioral syndromes between species based upon shared genetic variation. A model system in which to examine differences in correlated traits across species is the unisexual-bisexual mating complex consisting of the bisexual, parent species, the sailfin molly (*Poecilia latipinna*) and the Atlantic molly (*P. mexicana*), and the unisexual, gynogenetic Amazon molly (*P. formosa*). Amazon mollies are a clonal, all-female species of live-bearing fish that are the product of a hybridization event between *P. latipinna* and *P. mexicana* (Hubbs & Hubbs 1932; Dawley 1989). Sperm from one of the parent species is required by the Amazon molly to start embryogenesis (Hubbs &

Hubbs 1932). As such, Amazon mollies are considered sexual parasites of the host (i.e. parent) species.

Previous research has shown that Amazon mollies are more aggressive in a mating context than female sailfin mollies (Foran & Ryan 1994), which may be adaptive as this behavior is likely to increase the frequency of matings obtained by Amazon mollies (Schlupp et al. 1991; Foran & Ryan 1994). One study has examined differences in behavioral syndromes between Amazon molly and sailfin molly females (Scharnweber et al. 2011). This study did not detect differences in various boldness behaviors between the two species; however, they were unable to detect correlations among behaviors. Due to small sample sizes, the lack of behavioral syndromes or differences between the species may not have been detectable. Recent studies by Seda et al. (2012) and Muraco et al. (*in prep.*) have found evidence of behavioral syndromes in male sailfin mollies suggesting that they will also be found in female sailfin mollies.

In this study, we examined whether there were differences in behavioral syndromes between the unisexual, Amazon molly and females of the bisexual, sailfin molly. As Amazon mollies benefit from behaviors that increase the chance of obtaining any mates (i.e., they only need sperm to start the development of their eggs) rather than from high quality mates, we hypothesize that Amazon mollies will have a behavioral syndrome that results in them obtaining any possible matings, whereas female sailfin mollies will have a different behavioral syndrome as an outcome of benefitting from mating with higher-quality males. Therefore, we predict that Amazon mollies will have different behavioral syndromes than female sailfin mollies. Alternatively, given that Amazon mollies are a hybrid between sailfin mollies and Atlantic mollies, it is possible

that behavioral syndromes will not differ between species as a result of shared genetic variation.

We hypothesize that there will be a relationship between stress hormone (cortisol) production and behavioral type, and that this relationship will differ between species. Although there has been mixed support for a relationship between behavioral type and hormone production (Thomson et al. 2011; Seda et al. 2012; Muraco et al. *in prep.*), we predict that individuals with a bold behavioral type (and its correlates) should have higher baseline release rates of cortisol but a low cortisol response (Cockrem 2007; Carere et al. 2010; Muraco et al. *in prep.*). These results will provide novel insight into how this unique mating system might influence behavioral syndromes in females of both species, as well as the impact of genetic similarities on behavioral syndromes, and will provide a greater understanding of the proximate basis to, and ultimate consequences of, behavioral syndromes.

Methods

Fish Maintenance

We tested wild-caught female sailfin mollies, as well as Amazon mollies, that were collected from the headwaters of the San Marcos River, Spring Lake, San Marcos, Hays County, TX (29.89 N, 97.82 W) from May 2011-January 2012. This population of sailfin mollies has been sympatric with Amazon mollies for less than 100 years, as sailfin mollies were introduced in the 1930s and Amazon mollies were introduced in the 1950s (Brown 1953; Hubbs et al. 1953; Hubbs et al. 1991; Schlupp et al. 2002). We maintained fishes in the laboratory for a minimum of 30 days prior to experimentation to control for receptivity. We housed females in single-sex, mixed-species aquaria (38-L). We

maintained all fish on a 14:10 L:D cycle and fed them a combination of brine shrimp (*Artemia* spp.; Bio-Marine) and Spirulina Flakes mixed with Aquamax[®] Fry Starter 200 (PMI Nutrition International). We fed fish once daily between 1630 and 1730 h to control for any hormonal changes due to feeding.

Identification of Behavioral Types

The experimental design of this study follows that of Muraco et al. (*in prep.*), excluding the mate preference component. We tested individual females of each species (n=40 Amazon mollies; n=40 female sailfin mollies) in each of three treatments: (1) activity/boldness/exploration, (2) sociability, and (3) repeatability of activity, boldness, and exploration. Individuals experienced treatments in numerical order to compensate for the need to control the timing of hormone sampling. Individuals were given at least 1h between treatments.

Briefly, in treatment 1, we placed individual females into an opaque, acclimation chamber (16.5x15x13 cm) for 5 min within an experimental arena (70x40x10 cm), that was marked with a grid of 36 total squares (each 7.5x8.5 cm). We quantified *boldness* as (max time to exit)-(individual time to exit), where max time to exit totaled 600s. Additionally, we quantified *activity* as the total time (s) that individuals spent moving in the 5 min after emergence; *exploration* was calculated as the total number of grids that an individual entered during the same time period. In treatment 2, we placed individual females into an opaque, acclimation chamber (15 cm in diameter) for 5 min, and then we recorded their time spent near a mixed-species shoal as a measure of sociability. We tested multiple females simultaneously and recorded their behavior with EvoCam (by Evological) software. Individual, behavioral metrics were scored for each female from

the recorded videos. Finally, in treatment 3, we repeated treatments 1 and 2 for all 80 individual females. This allowed us to obtain measures of individual consistency and repeatability of behavior over two days. Measures of repeatability and consistency are necessary in studies of behavioral syndromes, as this framework assumes that individual expression of suites of correlated behaviors is stable over time.

Hormone Sampling

We obtained baseline cortisol release rates via water-borne hormone samples 24h before Treatment 1. Additionally, we obtained post-stimulus cortisol release rates via water-borne hormone samples after Treatment 1 and within \pm 1hr of the baseline sampling. Hormone samples were taken between 0800-1300h to control for circadian variation in hormone levels (Lorenzi et al. 2008). Water-borne hormone collection methods followed Gabor & Grober (2010). Briefly, individual females were placed into a 250 mL beaker filled with 100 mL of conditioned water and then removed after 1 hour. Water samples were stored at -20 °C until hormone assays were completed at a later date (Ellis et al. 2004). All subsequent hormone assay procedures followed the methods of Gabor & Grober (2010).

To assay cortisol levels, we used commercially available enzyme-immunoassay (EIA) kits (Cayman Chemical). Gabor & Contreras (provisionally accepted) validated the use of water-borne hormones to measure cortisol on EIA plates, as well as the correlation between water-borne release rates and plasma cortisol production for sailfin mollies. Here, we provide the validation for Amazon mollies. All samples were run in duplicate on 96-well plates and read by a fluorescent plate reader (BioTek Powerwave XS).

We validated the cortisol EIA kits (Cayman Chemical) for *P. formosa* using water samples from 10 non-experimental *P. formosa* that were collected and extracted using methods similar to those described above. We combined the re-suspended samples in a concentrated pool. We diluted the pool 1:2 for the serial dilutions and 1:10 for quantitative recovery.

We assessed parallelism of the serial dilution curve (run in duplicate) using the pooled control for *P. formosa*. We constructed the log-logit transformed dilution curve using average percent maximum binding and pg/ml concentrations for eight dilution samples (from 1:2 to 1:128 dilution). The dilution curve was parallel to the standard curve (comparison of slopes; $t = -1.0525$, $P > 0.05$).

We determined the quantitative recovery of the water-extracted hormones by spiking the pooled control for *P. formosa* with each of the eight standards and running an un-manipulated pooled control sample. Expected recovery concentrations were based on the known amount of cortisol in control samples. The minimum observed recovery for *P. formosa* was 88%. The slope of the observed vs. expected curve was 0.84, indicating a linear relationship between observed and expected ($F_{(1,7)} = 354.27$, $r^2 = 0.98$; $P < 0.0001$).

Data Analysis

Data did not meet assumptions of parametric analyses; therefore, we used non-parametric analyses. We combined the data for both species for the Principal Components Analysis (PCA), as well as in subsequent analyses which required component scores from the PCA. We analyzed the data separately by species in all other analyses. Additionally, the two body size metrics (mass & SL) were positively correlated

for both species (Spearman's rank correlation: *P. formosa*: $\rho=0.9705$, $p<0.0001$; *P. latipinna*: $\rho=0.8777$ $p<0.0001$). Therefore, to account for effects of body size on behavior, we divided the behavioral scores of each individual by the standard length (SL) of that individual. Furthermore, we were unable to obtain values for activity and exploration from individuals that did not exit the acclimation chamber in Treatment 1; therefore, these individuals ($n=6$ *P. latipinna*, $n=7$ *P. formosa*) were excluded from analyses. Repeatability was measured based on the raw behavioral data.

To identify behavioral syndromes, we used both Spearman's rank correlation, as well as with PCA. By definition, a behavioral syndrome consists of the correlation between two (or more) behaviors either within or among situations (Sih et al. 2004). Therefore, Spearman's rank correlation is adequate to identify behavioral syndromes (e.g. Bell 2005; Cote et al. 2010; Cote et al. 2011); however, PCA is most useful as a data exploration tool and variable reduction technique and is an important step when comparing behavioral syndrome composition between groups.

First, we used Spearman's rank correlation to examine whether there were significant, pair-wise correlations among the behavioral metrics. Because the pairs of behavioral metrics were correlated (Table 5), we examined whether there were multiple correlations among behaviors with PCA. We extracted individual component scores from the first two principal components (PC's) for both species. Behavioral metrics with a loading of 0.32 or greater were considered to be strongly associated with that component (Tabachnick & Fidell 1996). Additionally, we measured the repeatability of the activity, boldness, and exploration metrics from the intraclass correlation coefficient (ICC) based on variance components obtained from a one-way Analysis of Variance (ANOVA).

To examine differences in behavioral syndromes between the two species, we used a Kolmogorov-Smirnov test. The Kolmogorov-Smirnov test allowed us to test the null hypothesis that the distribution of PC scores, where the complete distribution of scores represents the behavioral syndrome and the individual points represent individual behavioral types, was similar between species. Therefore, rejection of the null hypothesis would suggest that the behavioral syndromes were different between species. We also used a *post-hoc* F-test to examine differences in the variance in PC scores between species. Finally, we used Fisher's Exact test allowed us to determine whether there was a difference in behavioral type frequency between species. In other words, rejection of the null hypothesis would suggest that the frequency with which a behavioral type occurred was dependent upon species.

To examine differences in stress hormone release rates between species, we used Spearman's rank correlation and a Mann-Whitney test. We took the natural logarithm (\ln) of the hormone measures and examined the correlation with activity, boldness, exploration, and PC scores for each species. We also examined difference in the baseline cortisol release rates (i.e. pre-stimulus) and the individual stress response (i.e. cortisol response of post-stimulus:pre-stimulus release rates) between species with a Mann-Whitney test. For all Mann-Whitney tests, $n=17$ for female sailfin mollies and $n=18$ for Amazon mollies. Analyses were performed on the transformed hormone data; figures show the untransformed hormone data.

Results

Identification of Behavioral Syndromes

For Amazon mollies, there were significant, pair-wise correlations among exploration-activity, sociability-activity, and sociability-exploration (Table 5). All other correlations were not significant. For female sailfin mollies, there were significant, pair-wise correlations among all behavioral metrics, except for exploration-boldness (Table 5). The four behaviors loaded onto two, orthogonal principal components that explained approximately 75% of the variance in the data set (Table 6). Based on the component loadings, principal component 1 (PC1) is positively associated with activity, exploration and sociability behaviors, whereas principal component 2 (PC2) is positively associated with boldness. Therefore, individuals with positive PC1 scores are more active, exploratory and social than individuals with negative PC1 scores (Figure 3). Additionally, individuals with positive PC2 scores are bolder than individuals with negative PC2 scores (Figure 3).

Repeatability of Behaviors

Individual females of both species were highly repeatable in their expression of active, bold and exploratory behaviors (Table 7).

Behavioral Syndrome Comparison between Species

There was a significant difference in the distribution of PC1 scores between species (Kolmogorov-Smirnov test: $D=0.3743$, $p=0.01$; Figure 3). Additionally, there was a significant difference in the distribution of PC2 scores between species (Kolmogorov-Smirnov test: $D=0.3209$, $p=0.04$; Figure 3). There was a significant difference between species in the frequency of behavioral types associated with PC1 (Fisher's Exact Test:

$p=0.03$; Figure 4); however, there was not a significant difference in the frequency of behavioral types associated with PC2 (Fisher's Exact test: $p>0.05$). Finally, there was not a significant difference between species in the variance of either PC1 or PC2 scores (PC1: $F_{33,34}=0.657$, $p>0.05$; PC2: $F_{33,34}=1.15$, $p>0.05$).

Behavioral Syndromes and Hormones

For female sailfin mollies, baseline cortisol release rates were significantly, positively correlated with exploration, and there was a trend towards significance with a negative correlation between the cortisol response and boldness (Table 8). There was a significant, positive correlation between boldness and baseline cortisol release rates, as well as a significant, negative correlation between boldness and the cortisol response, when individuals that did not exit were included in the analysis (Table 8). We did not detect any other significant correlations for female sailfin mollies. For Amazon mollies, there were no significant correlations between the hormone measures and any of the behavioral metrics (Table 8).

There was a significant difference in baseline cortisol release rates between species (Mann-Whitney Test: $Z=2.29$, $p<0.05$). On average, female sailfin mollies had lower baseline release rates of cortisol than Amazon mollies (Figure 5). Finally, there was a significant difference in the cortisol response between species (Mann-Whitney Test: $Z=-3.02$, $p<0.01$). Female sailfin mollies had a higher cortisol response to exploring a new area than Amazon mollies (Figure 6).

Discussion

Both the unisexual, hybrid Amazon mollies and the bisexual, female sailfin mollies showed evidence of behavioral syndromes in this sympatric population. As

predicted, these two species also differed in their behavioral syndromes with female sailfin mollies behaving primarily more active, exploratory and social than Amazon mollies. However, these two species did not appear to differ in boldness. The differences between the species in baseline cortisol production and overall cortisol response to exploring a new area may provide a proximate mechanism for these differences. Additionally, the hybrid origin of Amazon mollies may be the basis for these behavioral and hormonal differences between the species.

Evidence for Behavioral Syndromes

We found provided evidence of behavioral syndromes in this population consisting of Amazon mollies and female sailfin mollies. Amazon mollies exhibited significant, pair-wise correlations among activity, exploration and social behaviors (Table 5), whereas female sailfin mollies exhibited significant, pair-wise correlations among all behaviors except for the exploration-boldness comparison (Table 5). When the data for both species were combined, the behaviors loaded onto two orthogonal principal components (PC's) that explained approximately 75% of the variance (Table 6). Additionally, females of both species were highly repeatable in all behaviors (Table 7). Repeatability values found here are moderately high when compared to the values found in a meta-analysis conducted by Bell et al. (2009).

This study adds to the overall body of literature that supports the evidence of behavioral syndromes in nature, as well as adding to the growing support that behavioral syndromes are present in populations of sailfin. To date, two studies have provided evidence of behavioral syndromes in male sailfin mollies (Seda et al. 2012; Muraco et al. *in prep.*); however, as Scharnweber et al. (2011) was unable to detect correlations among

boldness behaviors, (possibly due to small sample sizes), ours is the first to demonstrate behavioral syndromes in females of both species in this unisexual-bisexual mating complex.

Species-level Differences in Behavioral Syndromes

We additionally provided evidence for differences in behavioral syndrome composition between two closely-related species. To support this claim, we found significant differences in the distribution of both PC1 and PC2 scores between species (Figure 3). Additionally, there was a significant difference between species in the frequency of behavioral types associated with principal component 1 (PC1; Figure 4). Specifically, female sailfin mollies appear to be active, exploratory and social than Amazon mollies, whereas the two species do not appear to differ in boldness traits (Figure 4). Scharnweber et al. (2011) also determined that there were not interspecific differences in boldness traits in these species. The lack of differences in boldness may be an outcome of shared genetic ancestry but further research is needed to confirm this hypothesis.

Our results did not support our prediction that Amazon mollies would be more active, bold, and exploratory than female sailfin mollies, as we predicted that these behaviors would be more likely to increase the likelihood of obtaining a mating from male sailfin mollies. Instead, the results presented here suggest the opposite, such that female sailfin mollies appear to be more active and exploratory (though not more bold) than Amazon mollies (Figure 4). In this study, we did not explicitly test whether females with a specific behavioral type were more likely to obtain a mating; therefore, an interesting follow-up would be to examine whether there is a relationship between

behavioral type and mate acquisition abilities both within and between females of both species in this unisexual-bisexual mating complex.

An alternative hypothesis for these differences between females is that females are exhibiting alternative reproductive strategies that differ between species. We originally predicted that being active, bold, exploratory and social would increase the chances of obtaining a mate. For example, Foran & Ryan (1994) found that Amazon mollies were more aggressive on average than female sailfin mollies, which might result in a higher frequency of matings via blocking of the female sailfin molly. However, given our results that Amazon mollies are less active, exploratory and social than female sailfin mollies, it might be the case that Amazon mollies are exhibiting a suite of submissive behaviors. The act of submission would be adaptive if it increases the probability of obtaining a mate, perhaps from smaller males that are likely to make mating mistakes due to decreased inspection time (Travis & Woodard 1989; Travis et al. 1990; Alberici da Barbiano et al. 2011). It would be interesting to compare the reproductive strategies of Amazon mollies in different social contexts, that is, in the presence of males when female sailfin mollies are present versus when they are absent.

Our study provides an interesting situation in which to question the role that genetic similarities play in shaping behavioral syndromes. For example, as the Amazon molly is a hybrid between a sailfin molly and an Atlantic molly (Hubbs & Hubbs 1932), it is possible that Amazon mollies would exhibit a behavioral phenotype that is intermediate to both parent species. An important follow up to our study would be to compare behavioral syndrome composition between female sailfin and Atlantic mollies and Amazon mollies. Additionally, our system allows for explicit testing of the adaptive

hypothesis, as geographic variation in behavioral syndrome composition would falsify the constraint hypothesis (Bell 2005).

Stress Hormones and Behavioral Syndromes

It is axiomatic that hormones regulate behavior in a variety of ways. Furthermore, there is increasing evidence that hormones and behavioral syndromes are linked, with particular emphasis on the role of stress hormones (e.g. Koolhaas et al. 1999; Archard et al. 2012; Aubin-Horth et al. 2012). Given that we found differences in behavioral syndrome composition between species, we explored the underlying hormonal differences as a basis for understanding the differences in behavioral syndromes. However, the relationship between stress hormone production and behavioral syndromes in our system is not clear. We found a significant, positive correlation between baseline cortisol release rates and exploration, as well as a negative trend between the cortisol response and boldness in female sailfin mollies (Table 8). However, when we included females that did not exit in Treatment 1, we found a significant relationship between baseline cortisol release rates, the cortisol response and boldness (Table 8). There was not a relationship between stress hormone measures and behavioral metrics in Amazon mollies. These results suggest that bold and exploratory individuals of female sailfin mollies have higher baseline release rates of cortisol than shy and non-exploratory individuals (Table 8). Additionally, bold individuals of female sailfin mollies tend to become less stressed, as they have a reduced stress response as compared to other such individuals (Table 8).

These results supported our hypothesis that bold individuals would have higher baseline release rates of cortisol, as these individuals are likely to deal with stressors

more effectively than others. For example, a study by Thaker et al. (2009) found that elevated levels of glucocorticoids can result in increased survival of individual tree lizards (*Urosaurus ornatus*) via enhanced anti-predator responsiveness. Therefore, it appears as though elevated levels of stress hormones may adaptively modulate behavior, as long as there is not chronic exposure to aversive stimuli.

Additionally, we have provided evidence that there are species-level differences in stress hormone production, which may account for the differences observed in the behavioral syndromes between the species. However, further testing is required to confirm this relationship. Our data are consistent with the hypothesis that Amazon mollies would have higher baseline release rates of cortisol than female sailfin mollies (Figure 5), even though the two species did not differ in boldness traits as predicted. Furthermore, we have shown that there was a difference in the stress response between species, such that female sailfin mollies had a higher cortisol response (post-stimulus:baseline) on average than did Amazon mollies (Figure 4). Chronic elevation of glucocorticoids (i.e. stress hormones) can result in a reallocation of resources from growth and reproduction to factors crucial to immediate survival (reviewed in Wendelaar Bonga 1997). Although Amazon mollies had higher baseline release rates of cortisol than female sailfin mollies (Figure 3), they also had a significantly lower stress response. If Amazon mollies are less stressed, or have a decreased stress response, in nature than female sailfin mollies, then this could provide an explanation as to why Amazon mollies tend to be larger than their bisexual counterparts (Gumm & Gabor 2005; Gabor & Aspbury 2008). Prior studies have shown that chronically-stressed individuals exhibit reduced growth patterns compared to less-stressed individuals (Barton et al. 1987). In

other words, Amazon mollies are more likely to grow larger than female sailfin mollies because they retain more resources for growth due to their diminished stress response.

Conclusions

When female sailfin mollies and Amazon mollies occur in sympatry, they are in direct competition for matings from the same population of males. However, both species may differ in mating preferences because Amazon mollies benefit from any matings not just high quality matings. These factors, in turn, could affect the behavioral syndromes found in each species. In this study, we discovered that female sailfin mollies and Amazon mollies exhibit different behavioral syndromes. Therefore, it is possible that females may differ in their mate acquisition abilities as a result of differences in their underlying behavioral type. Additionally, we found some evidence for a proximal link between boldness and exploration behaviors and stress hormones for female sailfin mollies. These results suggest that elevated, yet non-chronic, levels of glucocorticoids can regulate behavior in an adaptive fashion. Finally, we found that female sailfin mollies showed a higher cortisol response to the stress of a novel arena than Amazon mollies, but Amazons exhibited higher baseline release rates. These results could provide an explanation for why Amazons are, on average, larger than female sailfin mollies, such that Amazons may be able to utilize more resources for growth due to a reduced stress-response. In summary, the results presented here have provided evidence of a proximate link between stress hormones and behavioral syndromes, as well as a possible ultimate explanation for the differences in behavioral syndrome composition between species.

Acknowledgements

We thank G. Aron, F. Horne, R. Mclean, and C. Nice for loaning space and equipment needed in this study. We also thank the EEB discussion group at Texas State University-San Marcos for helpful comments on the manuscript. Finally, we thank members of the GASP lab, especially L. Alberici da Barbiano and C. Blake, for their help with catching and maintaining the fish. This work was funded by a National Science Foundation Grant, as well as a Grant-in-Aid of Research from Sigma Xi (to JJM). Fishes were collected in Texas under scientific collection permit no. SPR-0604-389. Our experimental protocol was reviewed and approved by Texas State University Institutional Animal Care and Use Committee (protocol 1032_0924_26).

Tables

Table 5. Spearman's rank correlation for each of the pair-wise behavioral comparisons between species.

Pair-wise Comparisons	<i>P. formosa</i>	<i>P. latipinna</i>
Boldness-Activity	0.1484	0.3448*
Exploration-Activity	0.4836**	0.5933**
Exploration-Boldness	-0.0137	0.2788
Sociability-Activity	0.4579**	0.5135**
Sociability-Boldness	0.1203	0.3506*
Sociability-Exploration	0.3834*	0.3455*

Table 6. Component loadings of behaviors onto principal components, as well as the variance explained by each component. Behaviors with a loading of at least 0.32 are considered to be associated with the component. Principal components identify multiple correlations among behaviors, i.e. behavioral syndromes.

Behavior	PC1	PC2
Activity	0.6205	-0.1490
Boldness	0.1854	0.9318
Exploration	0.5813	-0.2833
Sociability	0.4926	0.1713
Variance Explained (%)	50.10	24.95
Cumulative Variance Explained (%)	50.10	75.05

Table 7. Repeatability (intraclass correlation coefficient; ICC) of three behaviors over a two-day time period for each species. Individuals of both species were highly repeatable in all three behaviors.

	Activity	Boldness	Exploration
	ICC ± SE	ICC ± SE	ICC ± SE
<i>P. latipinna</i>	0.57 ± 0.15	0.65 ± 0.13	0.53 ± 0.16
	F _{39,79} =3.62, p<0.0001	F _{39,79} =4.72, p<0.0001	F _{39,79} =3.29, p=0.0001
<i>P. formosa</i>	0.61 ± 0.14	0.55 ± 0.16	0.47 ± 0.18
	F _{39,79} =4.09, p<0.0001	F _{39,79} =3.40, p<0.0001	F _{39,79} =2.76, p<0.001

Tables (continued)

Table 8. Spearman's rank correlation between individual behavioral metrics, composite behavioral metrics (PC scores) and measures of stress hormone (cortisol) production for each species.

Species	Behavior	Baseline	Cortisol Response^a
<i>P. latipinna</i> n=17	Activity	0.2353	-0.1912
	Boldness	0.3578	-0.4755 ^b
	Exploration	0.5294*	-0.4387
	PC1	0.3652	-0.2770
	PC2	-0.0147	0.1838
<i>P. latipinna</i> n=20	Boldness	0.5904**	-0.5407*
<i>P. formosa</i> n=18	Activity	-0.3643	0.1641
	Boldness	0.2941	-0.0279
	Exploration	0.1063	-0.0237
	PC1	-0.0939	0.0547
	PC2	0.3808	0.2982
<i>P. formosa</i> n=19	Boldness	0.2018	-0.0439

*p<0.05, **p<0.01

^aCortisol response = Post-stimulus/Baseline

^bp=0.054

Figures

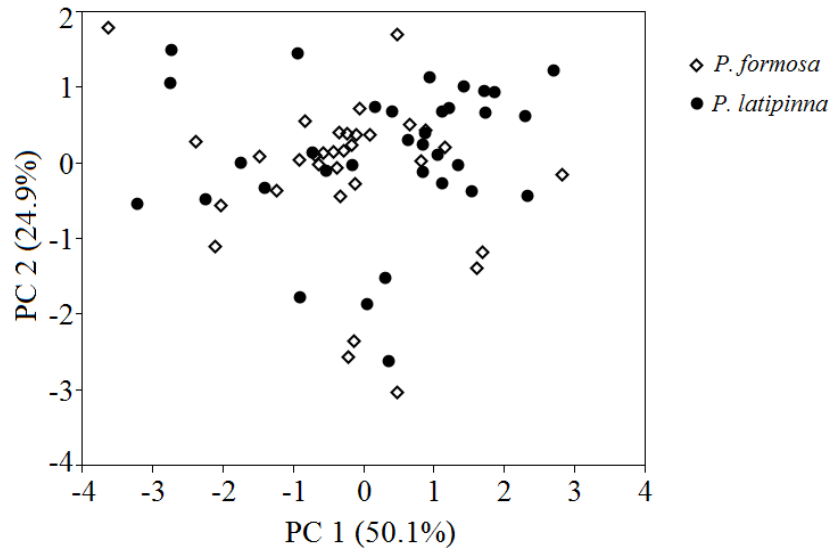


Figure 3. Biplot of the variable scores extracted from the first two principal components for each species.

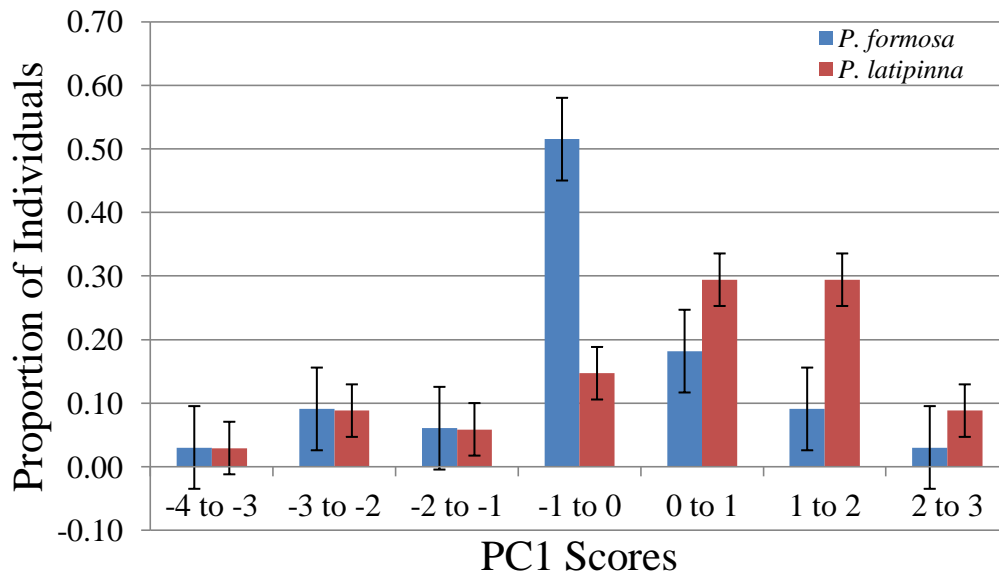


Figure 4. Difference in the proportion of individual behavioral types between species based on their PC1 score. Positive PC1 scores are associated with activity, exploration and sociability. Negative PC1 scores are associated with inactivity, non-exploration, and asociability.

Figures (continued)

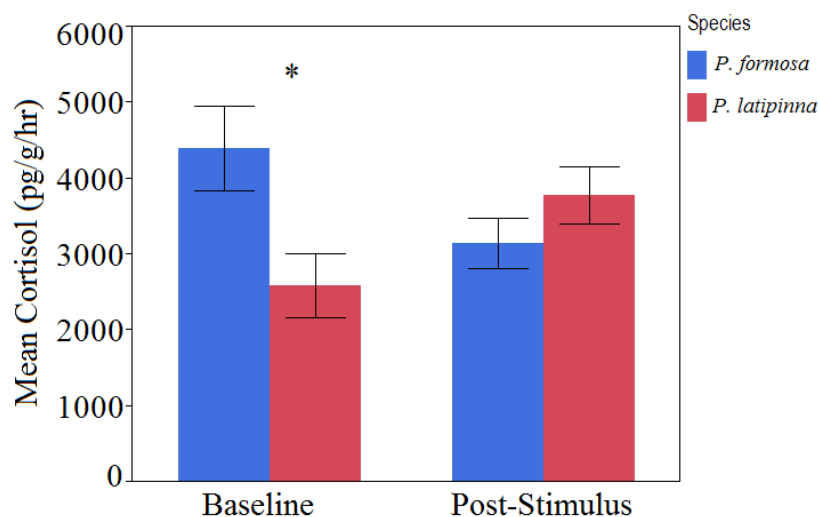


Figure 5. Average cortisol release rates ($\text{pg g}^{-1} \text{hour}^{-1}$) for each species. Baseline values represent cortisol samples taken prior to experimentation; Post-stimulus values represent cortisol samples taken after exposure to Treatment 1. There was a significant difference in baseline cortisol release rates between species, but there was not a significant difference in post-stimulus release rates. *indicates $p < 0.05$

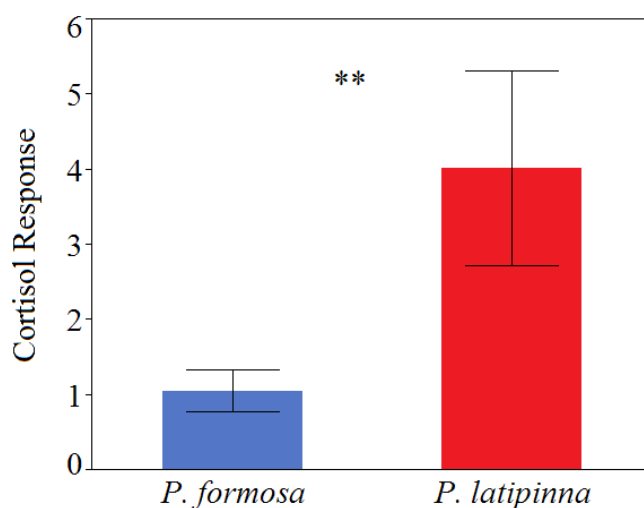


Figure 6. Difference in the cortisol response between Amazon mollies (*P. formosa*) and female sailfin mollies (*P. latipinna*). Cortisol response is calculated as post-stimulus release rate/baseline release rate per individual. Values greater than 1.0 indicate that individuals released more cortisol after exposure to Treatment 1 than was released prior to experimentation. There was a significant difference in the cortisol response between species. ** indicates $p < 0.01$

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