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SOCIAL BEHAVIOR, DOMINANCE, AND NEUROMOLECULAR RESPONSES DIFFER IN MALE EASTERN MOSQUITOFISH (*GAMBUSIA HOLBROOKI*) COLOR MORPHS

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Tanja Christine Zerulla

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To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Tanja Christine Zerulla, and entitled Social Behavior, Dominance, and Neuromolecular Responses Differ in Male Eastern Mosquitofish (*Gambusia holbrooki*) Color Morphs, having been approved in respect to style and intellectual content, is referred to you for judgment.

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DEDICATION

This dissertation is dedicated to my husband Brendan, our family, and our friends, who supported me in sickness and in health throughout this process; to my daughter Thea, who will grow up knowing that she can do hard things and accomplish whatever she sets her mind to; and to 10-year-old me, who wanted nothing more than to be the fish scientist that I am today.

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ABSTRACT OF THE DISSERTATION

SOCIAL BEHAVIOR, DOMINANCE, AND NEUROMOLECULAR RESPONSES DIFFER IN MALE EASTERN MOSQUITOFISH (GAMBUSIA HOLBROOKI) COLOR MORPHS

by

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Across taxa, morphological and behavioral traits can correlate within individuals. Eastern Mosquitofish (*Gambusia holbrooki*) exhibit a discrete color polymorphism. Males are either the common silver morph or the uncommon mottled morph, unpigmented with black blotches. I first reviewed and synthesized literature about the biology and correlated phenotypes of this color pattern in Poeciliidae. This review revealed conflicting results about the social behavior of mosquitofish morphs. I subsequently studied how behavior was affected by the focal male's morph and by indirect genetic effects, specifically the phenotypes of interacting individuals. I investigated how social behavior differed across three social contexts: with a group of females, in a male-male dyad, and in a mix-sex group. Mottled males are more assertive towards female groups than silver males, but not in mix-sex contexts. In dyads and mix-sex groups, mottled males dominate silver males, which results from silver males altering their behavior based on the opponent male's morph. In silver mosquitofish, size usually predicts social dominance, but its effect on the dominance of mottled males was unknown. Arranging social dyads of every color morph-combination across a range of size differences, I found that larger relative body size predicts dominance in within-morph conflicts, but morph is more important in between-morph conflicts. Mottled males dominate silver males, regardless of size differences. Finally, to determine potential genetic mechanisms for phenotypic correlations within mosquitofish morphs, I used whole-brain transcriptomics of mottled and silver males following interactions with a silver male. Morphs show distinct differences in their neuromolecular responses. I identify three differentially expressed transcripts that correlate with social behavior, including GIPC1 (GIPC PDZ domain containing family, member 1), which was previously determined to have a Y-chromosome linked allele associated with the mottled pattern. In conclusion, social behavior depends both on the male's morph and on the phenotypes of conspecific interacting individuals, but mottled males dominate silver males across size differences and social contexts. Future studies should examine whether and how the differentially expressed transcripts identified here affect the expression of color pattern and social dominance in male mosquitofish.

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CHAPTER 1

INTRODUCTION

Across vertebrates, morphological, physiological, and behavioral traits can correlate together within the individual as a suite of phenotypes (rev: Brockmann, 2001; Ducrest *et al.*, 2008; McGlothlin and Ketterson, 2008; McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018). Within populations, individuals can have different suites of correlated traits, which result in alternative phenotypes or morphs (Brockmann, 2001). The fitness of these alternative phenotypes or morphs is set by both internal processes, such as genetics, development, and physiology, as well as external processes like social and physical environment (Brockmann, 2001). Understanding phenotypic correlations holds the key to understanding evolution of alternative phenotypes. Color polymorphisms, in which two or more distinct color patterns exist in a population (Huxley, 1955), are especially popular study systems of phenotypic correlation because researchers can distinguish visually among the alternative morphs (McKinnon and Pierotti, 2010).

In the family Poeciliidae, color polymorphisms regularly correlate with other traits (Meffe and Snelson, 1989; Evans *et al*., 2011). Poeciliids are small and easy to capture, can be housed in large numbers in the lab, and have short generation times (Meffe and Snelson, 1989; Pyke, 2005; Evans *et al*., 2011). Because of these features, poeciliids are especially amenable for experiments on phenotypic correlations. In particular, multiple poeciliid species exhibit a discrete color polymorphism in which most fish are unspotted but a low frequency of individuals exhibit a melanic side-spotting pattern consisting of irregularly distributed black spots across the sides of their body (Myers, 1925; Zerulla and Stoddard, 2021). Because poeciliids are a well-studied taxonomic group for genetics, development, physiology, behavior, ecology, and evolution, I have proposed this polymorphic melanic side-spotting pattern as a potential model for studying phenotypic correlations (Zerulla and Stoddard, 2021).

In **Chapter 2**, I review the biology of the melanic side-spotting pattern across Poeciliidae. I describe the pattern and its inheritance in different species. I then comprehensively review and synthesize literature about phenotypes correlated with this pattern, from internal factors like genetics, development, and physiology to external factors like social and physical environment, as well as ecological selective pressures and possible evolutionary origins. Although I identify possible trends in phenotypic correlations, further research is required across all biological levels. I conclude the review by proposing a framework for future studies on the molecular and evolutionary mechanisms of phenotypic correlation for this polymorphic pattern.

Within poeciliids, Eastern Mosquitofish (*Gambusia holbrooki*) males exhibit this discrete color polymorphism; they express either the common silver pattern or the uncommon mottled pattern (first reported by: Myers 1925; Fig. 1). Mottled individuals can range in appearance from barely spotted to almost completely black (Regan, 1961). The mottled color pattern is sex-linked and highly heritable from the paternal line (Regan, 1961; Angus, 1989; Horth, 2006), indicating that differences in correlated traits between morphs have a genetic or epigenetic basis. Mosquitofish sex is easily determined because males have a modified anal fin ray that serves as an external mating organ, called the “gonopodium”, used for internal fertilization (Constantz, 1989). Furthermore, mosquitofish are found in high abundance (Pyke, 2005), so the uncommon mottled morph is more accessible than in other poeciliid species. Although mosquitofish have a peak breeding season in the summer, they can reproduce throughout the year, consistently providing new individuals for experiments (Pyke, 2005). Therefore, Eastern Mosquitofish represent an amenable system for studying phenotypic correlation.

Figure 1. Color morphs of the Eastern Mosquitofish (*Gambusia holbrooki*). The uncommon, mottled morph (left) is pale with irregularly distributed black and brown spots or blotches. The common, silver morph (right) is characterized by a pale olivaceous-silver color without spots.

Through direct genetic effects, an individual's genotype directly determines the phenotypic expression of both color pattern and correlated social behaviors (rev: Moore *et al*., 1997; Wolf *et al*., 1998). Melanin-based color patterns are associated with higher aggression and social dominance across taxa (rev: Ducrest *et al*., 2008; McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018). In the Bluefin Killifish (*Lucania goodei*), males with larger melanin outlines on their anal fins are socially dominant (Johnson and Fuller, 2015), while convict cichlid (*Amatitlania nigrofasciata*) females with darker melanic stripes and smaller orange spots are more aggressive (Schweitzer *et al*., 2015). In reptiles, black morphs of the Brazilian lizard *Tropidurus semitaeniatu*s exhibit more aggressive displays and were socially dominant over yellow morphs (Bruinjé *et al*., 2019). Similarly, Hermann's tortoises (*Eurotestudo boettgeri*) with darker shells are more aggressive towards other males (Mafli *et al*., 2011). In birds, larger melanin ornaments are often associated with higher aggression and social dominance (rev: Jawor and Breitwisch, 2003). Direct genetic effects likely play some part in the social behaviors expressed by Eastern Mosquitofish (*G. holbrooki*) morphs. Male siblings sired by the same temperature-dependent mottled fathers were raised in different temperature conditions so that individuals have the same genotype (mottled), but different expressed phenotypes (silver or mottled; Horth, 2003). These siblings exhibit different social behavior responses corresponding to their color pattern, even though all share the mottled gene(s), indicating that the correlated behaviors and coloration are conditionally co-expressed (Horth, 2003).

Social behavior responses also depend on the traits expressed by conspecific interacting individuals, which can be reciprocal traits (e.g., bidirectional effects of social behavior) or non-reciprocal (e.g., sex, body size, color pattern; Moore *et al*., 1997; Wolf *et al*., 1998; McGlothlin *et al*., 2010). In mix-color and mix-sex social groups, mottled males direct more aggression towards silver males than they receive in return (Martin, 1977; Horth, 2003), but morphs do not differ in aggression levels towards same-color males (Martin, 1977); thus, social behavior differences between morphs depend on the phenotypes of individuals present in their social groups. Whether mottled and silver males differ in sexual activity varied across study designs (Martin, 1977; Nelson and Planes, 1993; Karplus and Algom, 1996; Horth, 2003; Kraft *et al*., 2018; Humphrey, 2019; Culumber *et al*., 2020); it is possible that indirect genetic effects may influence whether color pattern correlates with sexual activity and partially explain these conflicting results. Finally, silver juveniles adjust their behavior more than mottled males based on prior experience and on the morph of adult males present in their mix-sex social groups (Kraft *et al*., 2018). Because the social behavior expressed by a morph both affects and is affected by the phenotypes of interacting individuals, mosquitofish morphs can experience different social environments, which in turn affects the fitness of and selective pressures experienced by each morph (Moore *et al*., 1997; Saltz and Nuzhdin, 2014; Kraft *et al*., 2016). Investigating the behavioral responses of silver and mottled males across different social environments is thus necessary to understand phenotypic correlation and its evolution within Eastern Mosquitofish morphs.

Previous studies investigated mosquitofish morph behavior in mix-sex social environments, but I observed male mosquitofish in the field also interacting for periods of time with only males or only females, and these contexts have not yet been systematically studied. In **Chapter 3**, I thus investigate how the expression of social behavior differs between silver and mottled male mosquitofish morphs depending on the interacting individual’s phenotype across different social contexts: (1) with female social partners, (2) with a silver or mottled male social partner, and (3) with both females and a silver or mottled male social partner. Mottled males direct more assertive attention towards females than silver males, but not when another male is present. Morphs do not inherently differ in male-directed aggression or submission; however, mottled males dominate silver males across contexts. This dominance is primarily driven by the decreased aggression and increased submission of silver males towards mottled males. Using a comprehensive ethogram of male Eastern Mosquitofish social behavior, I find that both morphs alter the specific behaviors that they express depending on the interacting fish’s phenotype and social context. Thus, the interaction of morph and social environment affects the expression of social behavior for male Eastern Mosquitofish morphs.

An interacting individual’s size also has the potential to strongly affect the expression of social behaviors by male mosquitofish morphs. Size usually predicts social dominance, but its effect on the dominance of mottled males over silver males is unknown. In **Chapter 4**, I observed conflicts in dyads of every color-combination across a range of size differences. Larger relative body size predicts dominance in within-morph conflicts, but color morph is more important in between-morph conflicts. Mottled males dominate silver males, regardless of size differences. Thus, social behavior of male mosquitofish morphs depends on the indirect genetic effects of multiple traits, but mottled males consistently dominate silver males.

Finally, the specific molecular pathways that regulate the expression of mosquitofish color patterns and its correlated traits are not known. As well, the specific gene that regulates the presence of the mottled color pattern has not been definitively identified (Kottler *et al.*, 2020). Therefore, I aim to identify possible genetic mechanisms underlying the Eastern Mosquitofish’s alternative phenotypes in **Chapter 5**. Past studies searching for genetic mechanisms of phenotypic correlation between melanin-based color polymorphisms and other traits have used a candidate gene approach, either by testing for expression differences in a single gene system between morphs or by manipulating a single gene’s function and observing the effect on expression of alternative phenotypes (San-Jose and Roulin, 2017). They often focused on melanocortins, pituitary peptide hormones that regulate melanin synthesis and distribution within a cell, as well as pleiotropically affect stress response, immunity, appetite, and social behaviors like aggression and sexual activity (rev: Ducrest *et al.*, 2008; San-Jose and Roulin, 2018). Unfortunately, mutations in the melanocortin system often explain only the color polymorphism and not the other correlated traits (San-Jose and Roulin, 2017). Both pigmentation and behavior are complex phenotypes that involve multiple genes across different systems, including other endocrine and neuroendocrine systems, developmental systems, transcription factors, and even possibly non protein-coding mechanisms like epigenetics (rev: Ducrest *et al.*, 2008; San-Jose and Roulin, 2017, 2020; Kottler and Schartl, 2018; Kottler *et al.*, 2020). Because of the likely complexity in regulation of the Eastern Mosquitofish's mottled pattern and its correlated behaviors, I focused on a systems biology approach wherein overall genetic response was recorded using RNA-sequencing of mottled and silver male brains after social interactions with silver males. Here I identify transcripts that are differentially expressed between morphs as well as a subset that may be involved in both the expression of color pattern and social behavior. Future functional gene studies could manipulate these systems to examine their effects on color pattern and on the behavioral responses of morphs to different phenotypes of interacting individuals.

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CHAPTER 2

The Biology of Polymorphic Melanic Side-Spotting Patterns in Poeciliid Fishes

2.1 Abstract

Melanin-based color patterns are an emerging model for studying molecular and evolutionary mechanisms driving phenotypic correlations. Extensive literature exists on color patterns and their correlated traits in the family Poeciliidae, indicating that these fishes are tractable models. We review the biology of polymorphic melanic side-spotting patterns characterized by macromelanophores forming irregular spotted patterns across fishes' flanks. These patterns are present in the genera *Gambusia*, *Limia*, *Phalloceros*, *Poecilia*, and *Xiphophorus*. Their presence is controlled by dominant genes on autosomes or sex chromosomes. Variation in expression is under polygenic control; however, these genes' identities are still largely unknown. In some *Gambusia holbrooki* and *Poecilia latipinna*, expression is dependent on low temperature exposure, but underlying molecular mechanisms are unknown. Spotted fish develop melanoma in rare cases and are a well-developed model for melanoma research. Little is known about other physiological correlates except that spotted *G. holbrooki* males exhibit higher basal cortisol levels than unspotted males and that metabolic rate does not differ between morphs in some *Xiphophorus* species. Behavioral differences between morphs are widespread, but specific to population, species, and social context. Spotted *G. holbrooki* males appear to be more social and more dominant. Juvenile spotted *G. holbrooki* have lower behavioral flexibility, and spotted *X. variatus* exhibit greater stress resistance. Findings conflict on whether morphs differ in sexual behavior and in sexual selection by females. Melanic side-spotting patterns are uncommon (<30%) in populations, although

extreme high-frequency populations exist. This low frequency is surprising for dominant genes, indicating that a variety of selective pressures influence both these patterns and their correlated traits. Little is known about reproductive life history traits. Spotted *G. holbrooki* are larger and have higher survival when uncommon, but underlying mechanisms remain unknown. Spotted morphs appear to have a strong selective advantage during predation. Predators prefer to attack and consume unspotted morphs; however, this preference disappears when spotted *G. holbrooki* males are common, indicating negative frequency-dependent selection. Spotted morphs are preferred socially under turbid conditions, but other environmental factors that shape phenotypic correlations and morph fitness have not been studied. Finally, we present questions for future studies on melanic side-spotting patterns.

2.2 Introduction

Poeciliidae are live-bearing fish of the sub-tropical and tropical waters of the Americas and Caribbean, a family that includes mollies, guppies, swordtails, platyfish, mosquitofish, limia, and others (Meffe and Snelson, 1989; Evans *et al*., 2011). Melanin polychromism has been observed in poeciliids since the late 1800's (Myers, 1925; Gordon and Gordon, 1957). These polymorphic patterns correlate with other traits, indicating that poeciliids have strong potential as a model taxonomic group for understanding the mechanisms, trends, and evolution of pigmentation-phenotype correlations common across the animal kingdom. A wealth of literature exists on poeciliid genetics, developmental biology, physiology, behavior, ecology, and evolution (for overviews, we recommend: Meffe and Snelson, 1989; Evans *et al*., 2011). Furthermore, poeciliids are an amenable group for laboratory and field experiments, being relatively easy to capture. Their small size and quick acclimatization to laboratory conditions, as well as their well-documented animal husbandry, makes them easy to maintain and handle for experiments. Poeciliids are also relatively easy to breed and have short generation times, which permits large-scale genetic and evolutionary studies. These features make them attractive over other taxa that may be more difficult to study, such as birds or mammals.

Previous reviews have focused on multiple color patterns and their related traits within a single species or genus of Poeciliidae (rev: Basolo, 2006; Culumber, 2014). Here we focus on a polychromism that has arisen repeatedly across poeciliid species: the melanic side-spotting pattern, in which fish develop irregularly distributed black spots across their flanks. We propose that this polychromism is a potential model for investigating trait correlation patterns across populations and species, as well as for understanding the molecular and evolutionary mechanisms that lead to these similar correlations. Specifically, we describe the development, inheritance, and genetics of the melanic side-spotting pattern. We review trends in correlations with physiological and behavioral traits, as well as ecological selective pressures and evolutionary origins that possibly shape the polychromism. Although the melanic side-spotting pattern exists in multiple poeciliid species, most research has been focused on the swordtails, genus *Xiphophorus,* due to their development as a laboratory model of melanoma, and on Eastern Mosquitofish (*Gambusia holbrooki*) because it is a common and easily accessible species. Consequently, a species bias is present in this review, so we recommend that any noted trends be thoroughly investigated in other poeciliids. Furthermore, most studies have not been repeated within a species and are limited to only a few populations or breeding lines within a species, so we encourage further studies to validate the limited trait correlations identified and presented in this review within and across species. We highlight what is still unknown about the melanic side-spotting pattern and suggest areas of further research throughout the review.

2.3 Mechanisms of Pigmentation-Phenotype Correlation

Correlations between color pattern and other phenotypic traits can arise through a variety of molecular and evolutionary mechanisms. The first potential evolutionary mechanism is **correlational selection**, in which similar selective pressures acting on separate genes results in a non-functional correlation between the melanic side-spotting pattern and another trait (Fig. 1A; rev: Sinervo and Svensson, 2002; Gray and McKinnon, 2007; Forsman *et al.*, 2008; McKinnon and Pierotti, 2010; Peiman and Robinson, 2017; San-Jose and Roulin, 2018). Correlational selection is rare (rev: McKinnon and Pierotti, 2010), as it usually leads to the second potential mechanism: **co-adaptive selection**. If these traits experience similar selective pressures, fish expressing both the pattern and the other trait would have increased fitness over those expressing one phenotype alone, so the separate genes for each trait are favored to be inherited together (Fig. 1B; rev.: Sinervo and Svensson, 2002; McKinnon and Pierotti, 2010). Alternatively, these separate genes can be inherited together if the fitness of the color pattern depends on the expression of the other trait or vice versa (**co-dependence**; Fig. 1C; rev.: Gray and McKinnon, 2007; Forsman *et al.*, 2008; Peiman and Robinson, 2017; San-Jose and Roulin, 2018), or if the color pattern and other trait must both be expressed and working together for a shared function (**co-specialization**; Fig. 1D, rev: Peiman and Robinson, 2017).

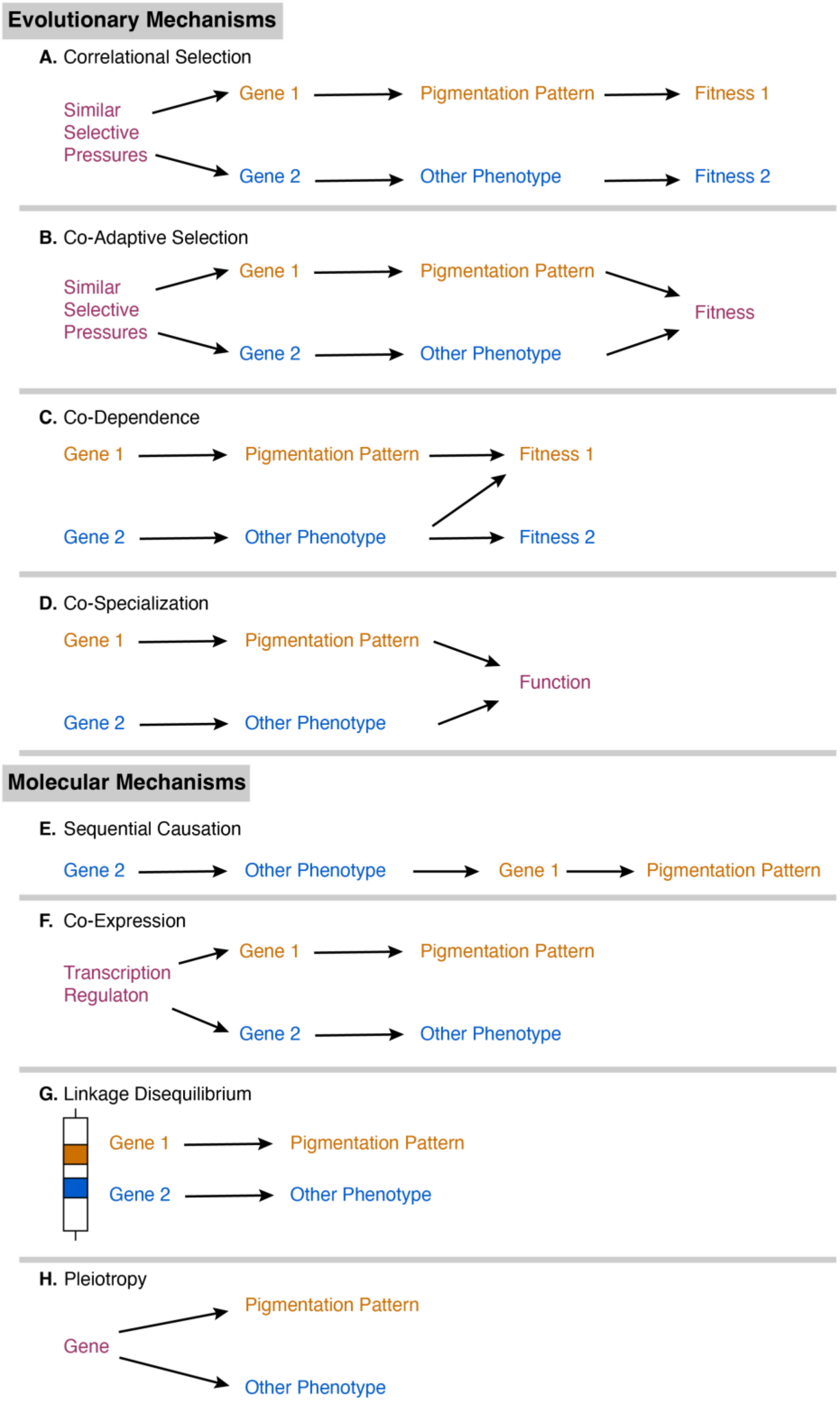

Figure 1. Diagram of proposed evolutionary mechanisms (**A-D**) and molecular mechanisms (**E-H**) of phenotypic correlation. Pathway component colors indicate whether they are specific to the pigmentation pattern (orange), specific to a correlated phenotype (blue), or function in both pathways (purple).

Correlations can also arise due to shared molecular mechanisms (rev: Ducrest *et al.*, 2008; McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018). Traits controlled by separate genes may be correlated through **sequential causation**, in which one trait is the precursor for another trait (Fig. 1E, rev: Peiman and Robinson, 2017). For example, a physiological change in hormones may be required first to initiate expression of the color pattern. Alternatively, these separate genes may have similar transcriptional regulation, such as through a shared promoter, that results in **co-expression** of the two different traits (Fig. 1F; rev: McKinnon and Pierotti, 2010). Genes that act on separate traits can also be inherited together if they are located close together on the same chromosome, as in **linkage disequilibrium** (Fig. 1G; rev: Sinervo and Svensson, 2002; Ducrest *et al.*, 2008; McKinnon and Pierotti, 2010; Peiman and Robinson, 2017; San-Jose and Roulin, 2018). For example, the *P* (*pituitary*) locus is associated with differences in sexual maturity and mating strategy in *Xiphophorus* (Kallman and Borkoski, 1978; Lampert *et al.*, 2010) and is close to the pigmentation locus on the Y chromosome (Kallman, 1983). Finally, genes and molecules rarely have a single biological function. Genes or molecular systems with multiple functions can thus affect multiple, separate traits through **pleiotropy** (Fig. 1H; rev: Sinervo and Svensson, 2002; Ducrest *et al.*, 2008; McKinnon and Pierotti, 2010; Peiman and Robinson, 2017; San-Jose and Roulin, 2018). The melanocortin system is a key candidate that has been proposed to pleiotropically regulate pigmentation and other traits (rev: Ducrest *et al.*, 2008; San-Jose and Roulin, 2018). Melanocortins are pituitary peptide hormones that regulate melanin synthesis, rapid distribution of pigment in a cell, glucocorticoid release, energy balance, food intake, aggression, and sexual activity. However, melanocortins are not the only possible mechanism for pleiotropy; many endocrine and neuroendocrine substances are involved in the regulation of both pigmentation and other traits, including other peptide hormones, amino acid hormones (e.g., thyroid hormone, GABA), monoamines (e.g., serotonin, dopamine), and sex steroids (e.g., testosterone). Neural crest cells have recently been proposed as a developmental mechanism for pleiotropy, as they differentiate into a variety of cells including pigment cells, neural cells, and endocrine cells (rev: Wilkins *et al*., 2014; San-Jose and Roulin, 2020). Determining which mechanism (or combination of mechanisms) underlies the specific correlations between melanic side-spotting patterns and other traits is not possible in a single study (San-Jose and Roulin, 2017), and will require multiple research groups working in parallel on different aspects of the biology of melanic side-spotting patterns and their correlated traits across different populations and species.

2.4 Melanic Spotting Patterns

2.4.1 Pattern Description

The base pigmentation pattern of poeciliids is a gray-olivaceous color, created by small pigment cells called micromelanophores outlining the fish's scales in a reticulate pattern (Gordon, 1927, 1928; Anders *et al*., 1984). Because this base pattern does not have any defined spots, we use the term "unspotted" interchangeably throughout this review (Fig. 2). Other color patterns overlay this base pattern, including those created by large pigment cells called macromelanophores (Gordon, 1927, 1928). This review focuses on what we term "melanic side-spotting patterns" that encompass a polychromism with a similar phenotype across poeciliid species and genera in which macromelanophores form irregularly distributed black spots on the fish's flanks (Fig. 2). These melanic side-spotting patterns can vary in amount and size of spots and in whether they are composed of punctate spots or spots that have coalesced into blotches (Fig. 2; Bellamy, 1936; Gordon and Gordon, 1957; Regan, 1961; Atz, 1962; Borowsky, 1973; Trendall and Johnson, 1981; Angus, 1983). To meet our criteria, the spotting pattern has to occur at minimum somewhere on the flank, but location of spotting on the flank can vary among species. While some species express spots over their whole body including head and fins (e.g., *Gambusia holbrooki*), spots in other species are restricted to specific sections of the flank, such as dorsally of the mid-lateral line (e.g., *Xiphophorus birchmanni*). We do not include micromelanophore or macromelanophore spotting patterns expressed only in fins, that form single or twin spots on the flank or caudal peduncle, or that form stripes or bars. These patterns either overlap with species that express the pattern as a fixed trait (e.g., stripe in *Heterandria formosa*) or have only been studied in depth for a single genus (e.g., tail spots on the caudal peduncle of *Xiphophorus*).

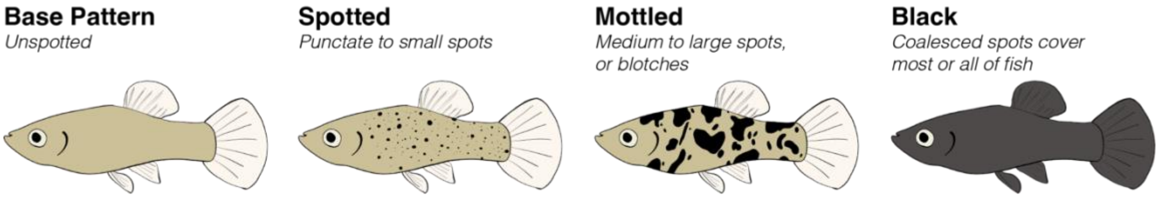

Figure 2. Representation of the poeciliid pigmentation patterns included in this review. The base pattern lacks macromelanophores, so we interchangeably refer to it as "unspotted." The melanic side-spotting patterns includes any pattern in poeciliid fishes that consists of macromelanophores forming spots that are irregularly distributed on the fish's flank. Included melanic side-spotting patterns may also extend into fins or be restricted to only a portion of the flank, not pictured here. The black morph is an extreme version of the spotting pattern.

Although phenotypically similar, melanic side-spotting patterns can differ from each other in terms of genetics, inheritance, sex, development, and organization, which is why some patterns have unique names to differentiate them in the literature (Table 1). We thus recommend that researchers always include images and specific descriptions and features of the pattern that include key terms like "side-spotted" to ensure that published literature is searchable and accessible for those not familiar with species-specific color patterns. Spotted morphs also have been referred to as melanic, melanistic, or black in the past; we advise that these terms be reserved for all-black morphs to avoid confusion, and recommend using descriptors like spotted, mottled, or blotched instead.

2.4.2 Pigment Cells

Melanophore differentiation has been reviewed extensively (rev: Anders and Anders, 1978; Vielkind and Vielkind, 1982; Anders *et al.*, 1984; Parichy and Spiewak, 2015). Briefly, all melanophores originate from neural crest cells (Humm and Young, 1956; Vielkind *et al.*, 1976, 1982). Pigment progenitor cells ("chromatoblasts") from the neural crest migrate across the body. These pigment progenitor cells then differentiate into melanophore progenitor cells ("melanoblasts"), which can either divide clonally or differentiate further. Melanophore progenitor cells differentiate irreversibly into either micro- or macromelanophore progenitor cells, which eventually differentiate terminally into micro- and macromelanophores (Fig. 3).

While micromelanophores differentiate in waves during embryonic stages to create the base pigmentation pattern, macromelanophores begin differentiation simultaneously and do so later in the adult fish's life to create spotting patterns (Humm and Young, 1956; Vielkind *et al.*, 1976, 1982). Macromelanophores are 150-300 µm in diameter, approximately three times the size of micromelanophores measured in *G. holbrooki* (Regan, 1961; Kottler *et al.*, 2020). Unlike micromelanophores, macromelanophores do not appear to have distance-dependent regulation, so they can overlap each other to form larger spots or blotches (Vielkind *et al.*, 1982; Anders *et al.*, 1984). Both melanophore types have dendritic or stellate shapes, but macromelanophores have thicker and more branched dendritic arms (Gordon, 1931b; Regan, 1961; Vielkind, 1976; Anders *et al*., 1984). Because they also contain higher numbers of melanosomes, organelles that synthesize and store melanin, macromelanophore spots appear intensely pigmented (Gordon, 1931b; Regan, 1961; Vielkind, 1976; Anders *et al*., 1984). When *G. holbrooki* skin is treated with adrenaline, micromelanophores aggregate their melanosomes so skin appears lighter, whereas melanosomes in macromelanophores do not move and remain distributed so spots stay dark (Kottler *et al*., 2020). This difference in adrenaline response suggests that some function of melanosome regulation is altered in macromelanophores (Kottler *et al*., 2020). Macromelanophores can inhibit differentiation and initiate destruction of micromelanophores, but not other pigment cell types (Gordon, 1928; Schmidt, 1978). Since destruction does not require direct cell contact, macromelanophores seem to be releasing an unidentified diffusible factor that specifically targets and eliminates micromelanophores (Schmidt, 1978). The function of this destruction is unknown, although it may serve to amplify the pattern as a visual signal by creating a halo effect around the spots (Schmidt, 1978; Vielkind *et al*., 1982).

2.4.3 Developmental Timeline

Poeciliid growth rate and age of sexual maturation is strongly influenced by environmental factors (Meffe and Snelson, 1989), so we describe development in terms of sexual maturation rather than time. Unspotted morphs are born without spots and remain unspotted for their entire life (Schröder, 1964; Angus, 1983). In the wild, poeciliids are born unspotted; spots often appear as secondary sexual characteristics begin to develop, such as the gonopodium in immature males, but the melanic side-spotting pattern is not fully established until after the fish is sexually mature, after which it can increase with age in some species (Bellamy, 1924, 1928; Myers, 1925; Gordon, 1931b; Regan, 1961; Atz, 1962; Kallman and Atz, 1966; Kallman, 1971; Menzel and Darnell, 1973; Angus, 1983; Martin, 1984; Franck *et al*., 1998; Angus *et al*., 1999; Meyer *et al*., 2006; Gutiérrez and García, 2007). For some spotted or all-black laboratory strains, spotted morphs can be born unspotted or spotted and either develop into spotted or completely black morphs after sexual maturity (Schröder, 1964; Angus, 1983).

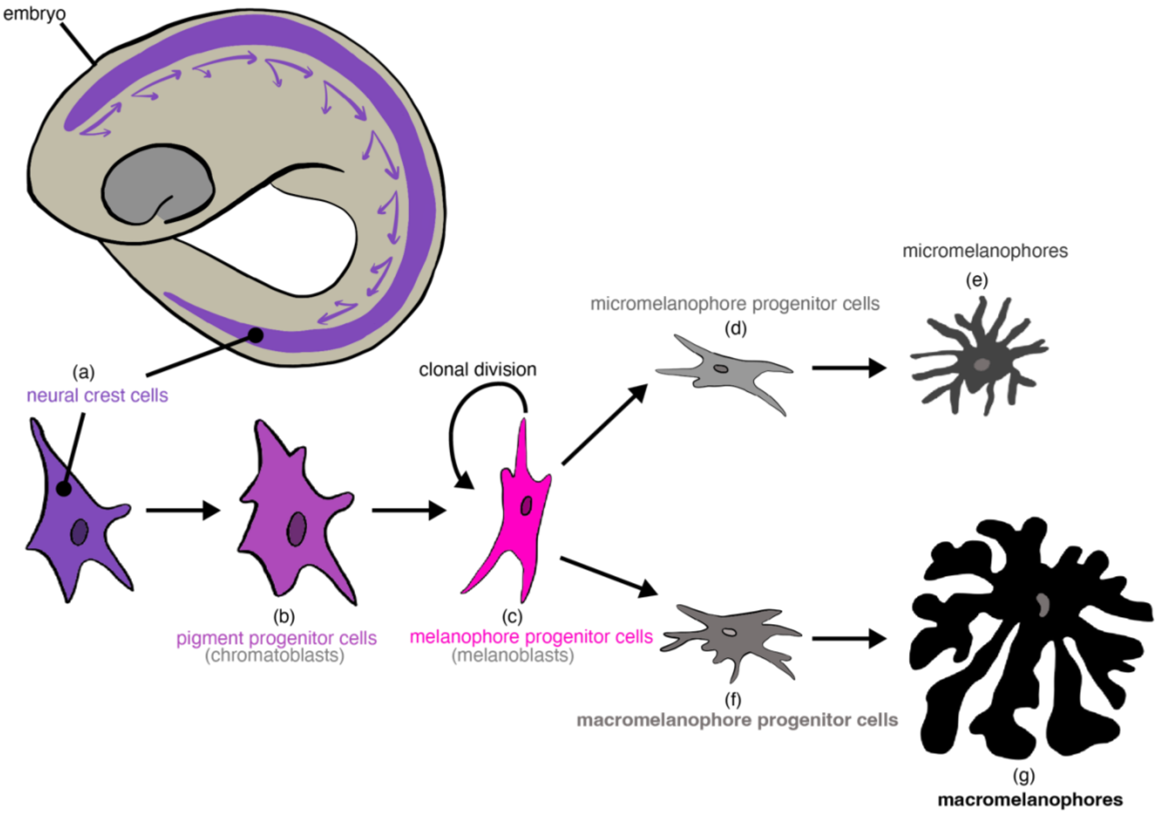

Figure 3. Differentiation of poeciliid melanophore cells. Neural crest cells (a) migrate in a ventral-posterior direction. They differentiate into pigment progenitor cells (b) that then further differentiate into melanophore progenitor cells (c). Melanophore propigment cells either divide clonally or terminally differentiate into micromelanophore (d) or macromelanophore progenitor cells (f), which differentiate terminally into micromelanophores (e) or macromelanophores (g), respectively.

2.5 Ecology

2.5.1 Frequency

For poeciliid species in which a melanic side-spotting pattern has been identified, the spotted morph is not present in every population (Gordon, 1948; Gordon and Gordon, 1957; Kallman, 1971; Martin, 1984; Horth, 2004; Culumber *et al*., 2014). When present, the frequency of spotted individuals varies depending on the population and species. In wild populations within their native range, spotted patterns ranged from a frequency of <0.5% to 28% of individuals in *Xiphophorus maculatus*, *X. helleri*, *X. variatus, X. cortezi, Gambusia holbrooki*, *Phalloceros caudimaculatus*, and *Poecilia mexicana* (Gordon, 1943, 1948; Gordon and Gordon, 1957; Regan, 1961; Kallman, 1971; Snelson *et al*., 1986; Karplus and Algom, 1996; Horth, 2004; Gutiérrez and García, 2007; Culumber *et al*., 2014). Rarely, extreme populations occur with higher frequencies of spotted morphs ranging from 40% to over 70%, as seen in *X. cortezi*, *X. variatus*, and *X. xiphidium* (Gordon, 1943; Kallman, 1971). Frequencies were often determined from a small subset of populations, and thus further sampling is required to understand frequency distributions for melanic side-spotting patterns across the entire native range of a species. Some invasive populations of *Poecilia latipinna* and *Phalloceros caudimaculatus* also exhibit frequencies >50%; however, these populations were founded by black or spotted individuals, which may partially explain the pattern's maintenance at a higher frequency (Nelson, 1983; McDowall, 1999; McNeil and Wilson, 2008; Petrescu-Mag *et al*., 2008; Koutsikos *et al*., 2017). Future comparisons of low-frequency and high-frequency populations may be interesting to investigate how population-level differences affect the frequency and maintenance of spotted morphs within a species.

Melanic side-spotting patterns likely arose through random, spontaneous mutations in the pigmentation pathway. Spontaneous mutations can maintain novel phenotypes at low frequencies within populations if the mutations occur at a similar or higher rate than they are selected out of a population (Gordon and Gordon, 1957; Angus, 1983; Horth, 2006; Gutiérrez and García, 2007); however, spontaneous mutation rates are very low, and the frequencies seen in most poeciliid populations would require an improbably high mutation rate, suggesting the wide taxonomic distribution and persistence of poeciliid side-spotting is instead maintained by other processes (Huxley, 1955; Horth, 2006; Gray and McKinnon, 2007; Forsman *et al*., 2008; McKinnon and Pierotti, 2010). Furthermore, melanic side-spotting patterns appear to be evolutionarily stable, as macromelanophore-based patterns have possibly existed within poeciliid species for millions of years (Kazianis and Borowsky, 1995). Frequency of spotted morphs often varies seasonally and annually by population, but ultimately stabilizes around a mean over time, indicating that the pattern is an evolutionarily stable phenotype (Gordon, 1943; Gordon and Gordon, 1957; Horth and Panayotova, 2012). Since melanic side-spotting persists stably across populations and species, various molecular constraints (e.g., pleiotropy) and selective pressures (e.g., predation) likely act upon these patterns and their correlated traits to maintain them at a low frequency in the population (Gordon and Gordon, 1957; Horth, 2004), and those that have been identified in the literature are reviewed in the following sections.

2.5.2 Life History Traits

Life history traits are important components of fitness, so differences between morphs reflect variation in the trade-offs as well as molecular, ecological, and evolutionary constraints that govern morph life history strategies, which in turn affect the frequency and maintenance of melanic side-spotting patterns. Unspotted and spotted female *Poecilia sphenops* have similar gestation times, regardless of the sire's pattern (Schröder, 1964). Although most *G. holbrooki* broods have equal sex ratios, some male-biased broods sired by spotted males have been observed (Horth, 2006). The spotted pattern locus could contain or be linked to a selfish gene element that directly or indirectly drives the production of spotted males, which would maintain the morph in some populations. Further research into brood sex ratios is required across populations and species. Other reproductive traits like fecundity, brood size, neonate size at birth, and energy investment during gestation by females have not been systematically investigated. *Xiphophorus* males with heavier pigmentation often have reduced survival (Koßwig, 1927; Mac Intyre, 1961), which could reflect a fitness disadvantage that drives down the frequency of spotted morphs. In *G. holbrooki*, spotted males are larger than unspotted males, which could give spotted males a dominance or mating advantage that contributes to the maintenance of this polymorphism (Martin, 1977; Horth *et al*., 2010; Culumber *et al*., 2018). However, this trend cannot be generalized to every population, as temperature-sensitive siblings of the same genotype but different phenotype were the same size (Horth, 2003), or to every species, since *X. helleri* morphs did not differ in body mass (Meyer *et al*., 2006). Although feeding behavior does not differ between morph juveniles in *G. holbrooki* (Culumber *et al*., 2018), other correlated physiological (e.g., growth rate) or behavioral (e.g., food competition) traits could still underlie the increased size of spotted males (Martin, 1977; Culumber *et al*., 2018), while also explaining why this size difference is not a general trait across populations and species. Further research into morph trade-offs among life history traits is required.

Life history traits do not vary due to genetic and physiological differences alone; variation in social and physical environment also influences these traits, so each morph's life history strategy may only be apparent in certain conditions. For example, different frequencies of spotted and unspotted morphs alter the social environment, as the likelihood of encountering and interacting with each morph will also be different. Juvenile body condition in *G. holbrooki* was better when individuals were raised with a preponderance of the opposite morph, which indicates negative frequency-dependent selection (Culumber *et al.*, 2018). Unspotted juveniles were affected to a greater degree, so morphs do seem to have underlying genetic or physiological differences that shape this trait (Culumber *et al.*, 2018). Survival rates also differed based on the frequency of the spotted morph in *G. holbrooki* (Horth and Travis, 2002). When spotted males were uncommon, they survived better than unspotted males; however, as frequency of spotted males increased, their survival decreased to the same level as unspotted males (Horth and Travis, 2002; Horth, 2004). The spotted morph thus seems to have a survival advantage when uncommon, again indicating negative frequency-dependent selection. Furthermore, higher frequencies of spotted males led to higher mortality of juveniles and females (Horth and Travis, 2002), which can lower a population's reproductive capacity. Altered behavior in the presence of spotted males is hypothesized to drive this lower survival (Horth and Travis, 2002). For example, juvenile feeding was lower in the presence of spotted males compared to unspotted males (Culumber *et al.*, 2018). The effect of other social environmental factors like density or sex ratio and of physical environmental factors on morph life history traits remains to be investigated across species.

2.5.3 Predation

Predation is a major selective pressure on animal color patterns (rev: Endler, 1988). Studies have found that predators prefer unspotted over spotted poeciliid morphs, which may contribute to a selective survival advantage for melanic side-spotting patterns. Pike topminnow (*Belonesox belizanus*) ate more unspotted than spotted *X. helleri* and aimed more hunting behaviors towards unspotted model fish than spotted (Dürr, 1996 and Becker, 1997 in: Franck *et al*., 2001). Similarly, sunfish (*Lepomis* sp.) ate more unspotted than spotted *G. holbrooki* (Martin, 1977; Bonner, 1980 in: Martin, 1986), while both dragonfly naiads (*Libellulid* sp.) and crayfish (*Procambarus* sp.) tended to eat the unspotted morph first (Horth, 2004).

Predation is hypothesized to maintain melanic side-spotting patterns at low frequencies in poeciliid populations through negative frequency-dependent selection (Gordon and Gordon, 1957; Martin, 1977; Horth, 2004). At a low frequency of spotted *G. holbrooki*, Largemouth Bass (*Micropterus salmoides*) ate more unspotted males (Horth, 2004). This preference disappeared when the bass had access to a higher frequency of spotted males, indicating that the spotted morph only has a selective survival advantage when it is uncommon (Horth, 2004). A lack of preference by bass when the morphs were equally present (Humphrey, 2019) could be because the spotted morph frequency was above a selection threshold for these predators. Modeling negative frequency-dependent predation of *G. holbrooki* morphs confirmed its role in stabilizing the pattern polymorphism in populations (Horth and Panayotova, 2012).

This advantage at low frequencies could result from predators using a search image (Endler, 1988), in which they seek unspotted fish as their major prey and overlook or ignore spotted fish because they are not a common and reliable food source. When the spotted morph becomes more common, the predators adjust their search images to include them, and the former advantage disappears (Horth, 2004). Alternatively, spotted morphs could have an advantage due to other correlated traits that have not yet been identified, such as anti-predator behaviors (Horth, 2004). In a preliminary experiment, spotted and unspotted *G. holbrooki* males did not differ in their response to predator odorants (Kraft, 2016). Both morphs also exhibited similar decreases in mating behavior and increases in freezing behavior when a restrained Largemouth Bass was present (Humphrey, 2019). Although *G. holbrooki* morphs from a single population do not appear to exhibit differences in anti-predator behavior, this result may differ by population, poeciliid species, and predator species. Crypsis may be more important in altering predation rate. Black mollies prefer shoals that were against a black background over shoals against a white background, suggesting that background matching is important for poeciliid color morphs (Bradner and McRobert, 2001a), but crypsis has not been studied in relation to the melanic side-spotting pattern.

2.5.4 Physical Environment

If environmental factors differentially affect the fitness of spotted and unspotted morphs, then frequency of the melanic side-spotting pattern may vary temporally and spatially with these factors (Gordon and Gordon, 1957; Horth and Travis, 2002). Some *X. helleri* live in seasonally variable streams that are clear in the dry season and turbid in the rainy season, while *Poecilia mexicana* populations are found in both non-sulfidic springs with clear water and in sulfidic springs with cloudy water that creates a white background (Franck *et al*., 2001; Culumber *et al*., 2014). Both unspotted *X. helleri* females in clear water and unspotted *P. mexicana* females from non-sulfidic springs preferred unspotted social partners (Franck *et al*., 2001; Culumber *et al*., 2014). In turbid water, unspotted female *X. helleri* switched their preference to spotted social partners, while unspotted female *P. mexicana* from cloudy sulfidic springs showed no preference between morphs (Franck *et al*., 2001; Culumber *et al*., 2014). In both species, spotted morphs preferred spotted social partners (Franck *et al*., 2001; Culumber *et al*., 2014). Spotted fish therefore seem to have a social advantage in low visibility conditions, most likely driven by the increased visibility of spotted individuals compared to unspotted (Franck *et al*., 2001). Since spotted *P. mexicana* were only obtained for sulfidic springs, these populations may alternatively differ in genetic preferences (Culumber *et al*., 2014). Selective advantage of the spotted morph thus seems to vary spatially and temporally depending on environmental conditions (Franck *et al*., 2001), and could explain different frequencies of the pattern across species' populations.

Environmental factors can also affect the expression of melanic side-spotting patterns (Regan, 1961; Schröder, 1964; Anders and Klinke, 1965). For example, a small number of populations of *G. holbrooki* and *Poecilia latipinna* are temperature-sensitive, exhibiting greater expression of the spotted pattern at low water temperatures experienced during the winter (<22 °C; Schröder, 1964; Angus, 1983, 1989; Angus *et al*. 1999; Horth, 2006). In simulated population models of *G. holbrooki*, temperature affected population frequency of the spotted morph both seasonally and long-term under different climate change scenarios (Horth and Panayotova, 2012; Panayotova and Horth, 2018). Variation in temporal expression of the spotted pattern could then influence how and when selective pressures affect this morph’s fitness. A fish born in the spring will be unspotted, then mature and reproduce during the summer, all before winter temperatures induce expression of the spotted phenotype (Angus, 1983). The spotted pattern gene(s) would thus be inherited and maintained in the population regardless of fitness costs later in the fish’s life because natural selection did not act upon the phenotype before the bulk of reproduction took place (Angus, 1983). Alternatively, the spotted and unspotted morphs may differ in fitness in warm and cool waters, possibly through other correlated traits, which would favor inducible phenotypic expression (Horth, 2003; Meyer *et al.*, 2006). The effect of other physical factors on pattern expression and morph fitness remains unexplored, including oxygen conditions, salinity, precipitation, seasonal flooding and drying out of areas (hydroperiod), habitat fragmentation, vegetative cover, food availability, ultraviolet light exposure, behavioral thermoregulation, and parasitism (Gordon and Gordon, 1957; Schröder, 1964; Borowsky, 1973; Nelson and Planes, 1993; Meyer *et al.*, 2006; Ducrest *et al.*, 2008; Petrescu-Mag *et al.*, 2008; Horth *et al.*, 2013; San-Jose and Roulin, 2018).

2.6 Social Behavior

2.6.1 Social Conflicts: Aggression and Dominance

Behavioral studies on the melanic side-spotting pattern exhibit a strong species bias to Eastern Mosquitofish (*Gambusia holbrooki*), likely due to its widespread distribution and accessibility. Behavioral differences between *G. holbrooki* morphs are easily observable, and had already been noted in wild populations a century ago (Myers, 1925), which likely further sparked the multitude of studies aimed at understanding spotted morph behavior.

Below we present trends in social behavior, but we advise caution in generalizing these trends and encourage further research into behaviors correlated with the melanic side-spotting pattern in other poeciliid species.

Melanin-based color patterns are associated with higher aggression, bold behavior, and social dominance across taxa (rev: Ducrest *et al*., 2008; McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018). In *G. holbrooki*, one morph is not inherently more aggressive than the other; rather, male *G. holbrooki* morphs alter their behavior depending on their social partner and the composition of their social group (Martin, 1977; Horth, 2003; Kraft *et al*., 2018). In same-morph groups, unspotted males exhibited higher aggression towards each other than spotted males did (Martin, 1977). In mixed-morph groups, unspotted and spotted males directed similar levels of aggression towards unspotted males, but unspotted males directed less aggression back to spotted males (Martin, 1977; Horth, 2003; Kraft *et al*., 2018). Spotted male dominance over unspotted males thus seems to be driven by behavioral changes of unspotted males (Martin, 1977; Horth, 2003; Kraft, 2016). A dominance advantage for spotted males could maintain the pattern in populations if it also confers a fitness advantage. However, this correlation may not be present in all poeciliids. Within male *X. helleri* dyads, neither unspotted nor spotted males achieved dominance over the other (Franck *et al*., 2001). As well, spotted *X. variatus* were more shy in new environments (Culumber, 2016), rather than bold as would be expected of dominant fish.

Unspotted *G. holbrooki* juveniles exhibited more submissive behaviors toward spotted adults than unspotted adults, whereas spotted juveniles did not change their behavior based on color composition of the social group (Kraft *et al*., 2018). Unyielding dominance behaviors by spotted males could be a tactic to win more social conflicts against unspotted males, as the unspotted males would be more likely to switch to submissive behaviors and thus lose. This behavioral inflexibility may also confer stress resistance, which is typically a fitness advantage (rev: Wingfield and Sapolsky, 2003), as the spotted morph would maintain their normal behavior in a variety of contexts. For example, spotted *X. variatus* exhibited greater behavioral resistance to stress than unspotted morphs (Culumber, 2016). Alternatively, this inflexibility may be a fitness disadvantage if spotted morphs do not avoid stressful or costly conflicts.

2.6.2 Precopulatory Sexual Selection: Mating and Sexual Activity

Findings conflict on whether morphs differ in sexual behavior and in precopulatory sexual selection by females. Where differences do exist, they seem to be influenced by population, species, morph frequency, experience, and environmental factors. (The reader interested in larger patterns may safely skip to the next section, *2.6.3 Social Groups*). In *G. holbrooki*, spotted males sometimes exhibited lower sexual activity than unspotted males (Nelson and Planes, 1993; Kraft *et al*., 2018), no difference (Martin, 1977; Karplus and Algom, 1996), or higher sexual activity (Martin, 1977; Karplus and Algom, 1996; Horth, 2003; Humphrey, 2019; Culumber *et al*., 2020). Results may depend on which specific sexual behaviors are measured. For example, Karplus and Algom (1996) found that morphs did not differ in time spent following females but spotted males were more likely to associate with females and interfere with other individuals' sexual interactions. These conflicting results may also be a function of different densities and morph frequencies (Martin, 1977), or selective pressures particular to the origin population that strongly influence the presence and direction of the pigmentation-behavior correlation.

Female behavior towards males can influence mating success for both courtship and coercive mating strategies in poeciliids (Bisazza *et al*., 2001). Female preferences for these morphs vary widely, most likely because responses are shaped by female morph identity, population, experience, and social context. For example, female *G. holbrooki* either directed more aggression towards spotted males and avoided them (Martin, 1977; Taylor *et al*., 1996), or were equally aggressive to both morphs (Horth, 2003). Female *G. holbrooki* preferred free-swimming unspotted males (Nelson and Planes, 1993; Taylor *et al*., 1996; Horth, 2003), but showed no preference when a barrier separated the sexes (Martin, 1986; Nelson and Planes, 1993) and have even preferred spotted and black stationary models (Gould *et al*., 1999). Even if *G. holbrooki* females have an innate preference for spotted males, other traits that are correlated with the melanic side-spotting pattern, such as sexual activity, may be more important in setting these preferences in the wild. Local environmental selective pressures may also strongly influence morph preferences because female preferences from different origin populations or environmental conditions differ in several species: *G. holbrooki* (Bisazza and Pilastro, 2000; Bisazza *et al*., 2001), *X. helleri* (Franck *et al*., 2001), and *Poecilia mexicana* (Culumber *et al*., 2014). Finally, it is unknown whether females prefer traits that only sometimes correlate with the melanic side-spotting pattern instead of the pattern itself, which may also explain these conflicting results.

Higher sexual activity or female preference for a color morph could lead to higher mating success and thus higher reproductive fitness, which would contribute to that morph’s maintenance in a population (Martin, 1977). In contrast, higher sexual activity could be disadvantageous if the males over-harass and stress females, leading to increased female mortality or decreased reproductive output (Horth and Travis, 2002). *G. holbrooki* females paired with size-matched spotted and unspotted males produced broods with frequencies of each phenotype that matched the expectation that neither morph had a mating advantage that translated into a fitness advantage (Horth *et al.*, 2010); however, reproductive fitness may depend on morph frequencies in a population and should still be investigated. To our knowledge, postcopulatory sexual selection has not been studied in species with side-spotted morphs.

2.6.3 Social Groups

Spotted and unspotted morphs do not encounter and interact with color morphs and sexes at similar rates. Each morph may thus have a different social group, which in turn affects social dynamics. Both *G. holbrooki* morphs interacted with the same number of unspotted males, but spotted males had more social partners and more interactions with females than unspotted males in the lab and in the wild (Kraft *et al.*, 2016). Both white and black mollies preferred phenotypically similar shoal mates (McRobert and Bradner, 1998; Bradner and McRobert, 2001b). Although white and black mollies are the result of domestication and hybridization among *Poecilia* species (McRobert and Bradner, 1998; Bradner and McRobert, 2001b), black mollies represent an extreme form of spotting and may still provide some insights, but these preferences should still be investigated in *Poecilia* spotted morphs. In clear water, unspotted and spotted female *X. helleri* also preferred phenotypically similar schools (Franck *et al.*, 2001). Asymmetric social interactions between morphs could lead to assortative schooling, influencing dominance and mating dynamics, which would in turn affect morph fitness. However, social groups are also influenced by experience, as both black and white mollies preferred to shoal with the morph with which they were raised over phenotypically similar fish (Ledesma and McRobert, 2008). Given that spotted morphs are usually uncommon, both morphs may prefer to shoal with the familiar and common unspotted morph, and thus assortative schooling and mating may not occur in the wild. To our knowledge, social interactions with heterospecific individuals have not yet been investigated.

2.7 Genetics

2.7.1 Modes of Inheritance

The melanic side-spotting pattern is always a genetically dominant trait (Gordon, 1927, 1937; Fraser and Gordon, 1929; Gordon and Smith, 1938; Gordon and Gordon, 1957; Atz, 1962; Schröder, 1964; Zander, 1965; Kallman and Atz, 1966; Kallman, 1971; Angus, 1983, 1989; Horth, 2006; Gutiérrez and García, 2007). Long-term persistence of a dominant trait indicates that multiple positive and negative selective pressures likely act upon the pattern and its correlated traits (Gordon and Gordon, 1957). Mode of inheritance differs across species and for each specific melanic side-spotting pattern (Table 1). In some species, the pattern is controlled by one or more autosomal genes (Atz, 1962; Schröder, 1964; Zander, 1965; Kallman and Atz, 1966; Kallman, 1971; Angus, 1983). In others, the patterns are linked to a sex chromosome (Gordon, 1927, 1946, 1951; Fraser and Gordon, 1929; Bellamy, 1936; Regan, 1961; Anders *et al.*, 1973; Kallman, 1983; Angus, 1989; Horth, 2006; Fernandez and Bowser, 2008). Sex-linkage may evolve in certain species through sexually antagonistic selection (Kottler and Schartl, 2018); the melanic side-spotting pattern or its correlated traits may be advantageous to male fitness but disadvantageous to female fitness, resulting in strong selective pressure for pattern expression to be greater in males than females (Kottler and Schartl, 2018). For melanic side-spotting patterns that are primarily expressed in males, spotted females are rare (Fraser and Gordon, 1929; Gordon, 1947; Snelson *et al*., 1986). These spotted females could arise through sex chromosome crossover, which is also a rare event (Kallman, 1965). Alternatively, these exceptional females have atypical sex determination and are genetically male, as was likely the case for a rare spotted female *G. holbrooki* (Snelson *et al*., 1986; Angus, 1989).

2.7.2 Polygenic Control

The melanic side-spotting pattern is heritable, controlled primarily by genetics (Koßwig, 1929). At least one gene exists that controls the presence of macromelanophore pigmentation, and this locus has been dubbed the *Macromelanophore-determining locus* or *Mdl* (Vielkind, 1976; Vielkind *et al*., 1976, 1982; Anders and Anders, 1978; Weis and Schartl, 1998; Gutbrod and Schartl, 1999). In some species, degree of pigmentation expression may be the result of gene dosage, the number of pigmentation alleles inherited (Schröder, 1964; Kallman, 1971; Gutiérrez and García, 2007, 2011). In others, specific melanic side-spotting patterns can vary in amount and size of spots, in developmental timing of expression, and in location of spots on the fish’s body (Table 1), indicating that spotting patterns have different genetic origins and/or that additional regulatory genes play a role in determining pattern expression (Gordon, 1931b; Kallman and Atz, 1966). Hybridizations across populations and species of *Xiphophorus* result in altered pattern expression, usually leading to heavier pigmentation and sometimes to melanomas (Koßwig, 1929; Gordon and Smith, 1938; Gordon, 1951; Atz, 1962, 1962; Anders *et al*., 1973). This altered pigmentation is the result of mismatches between the pattern genes of one population or species and the regulatory genes of another, indicating that these patterns are under polygenic control (Gordon and Smith, 1938; Gordon, 1951; Atz, 1962; Anders *et al*., 1973). The number of genes and the location of the loci involved may differ across poeciliid populations and species (Anders *et al*., 1973). One to a few genes control the pattern in some species (Schröder, 1964; Kallman, 1971; Horth, 2006), while others involve multiple genes across chromosomes interacting together (Kallman and Atz, 1966; Anders *et al*., 1973; Borowsky, 1973).

2.7.3 Candidate Genes for Melanic Side-Spotting Patterns

Most genetic work on macromelanophore pigmentation patterns has been conducted in the *Xiphophorus* melanoma model. Knowledge about the genetics underlying these patterns outside of *Xiphophorus* is practically non-existent with the notable exception of *Gambusia holbrooki* (Kottler and Schartl, 2018; Kottler *et al*., 2020). At this time, no gene has been explicitly identified as the macromelanophore-determining gene in poeciliids (Kottler *et al*., 2020). Candidate genes for the development and regulation of melanomas are reviewed elsewhere (rev: Schartl, 2008; Schartl and Walter, 2016), so we will focus on the genetic mechanisms that appear to be related to the presence and expression of melanic side-spotting patterns.

The presence of macromelanophores is controlled by the *Mdl* in *X. maculatus* and in *G. holbrooki* (Vielkind, 1976; Vielkind *et al*., 1976, 1982; Anders and Anders, 1978; Weis and Schartl, 1998; Gutbrod and Schartl, 1999; Kottler *et al*., 2020). This locus is hypothesized to consist of co-dominant alleles, including macromelanophore and other regulatory genes (Kallman and Atz, 1966; Anders *et al*., 1973; Vielkind and Vielkind, 1982; Weis and Schartl, 1998). The “macromelanophore gene(s)” that control the presence or absence of the melanic side-spotting pattern likely act on the melanophore progenitor cells, affecting whether they are assigned to the micro- or macromelanophore fate (Vielkind and Vielkind, 1982). In *G. holbrooki*, the presence of the spotted pattern was linked to an allele on the Y chromosome for *GIPC1* (*GIPC PDZ domain containing family member 1*), which functions in vesicle trafficking and sorting proteins to melanosomes for pigment synthesis (Liu *et al*., 2001; Kottler *et al*., 2020); however, its specific function in the melanic side-spotting pattern has not yet been determined. Because of its role in macromelanophore proliferation during melanoma formation, the *Xiphophorus* "macromelanophore gene" was thought to be X*mrk* (*Xiphophorus* receptor tyrosine kinase), a homolog of *epidermal growth factor receptor b* (Wittbrodt *et al*., 1989; Schartl, 2008). However, X*mrk* is found in species both with and without the spotting pattern, so it does not define the pattern's presence (Weis and Schartl, 1998; Schartl, 2008). At this time, X*mrk*'s known relation to spotting is as a marker of *Mdl*, although it may still have an undefined function related to pattern expression; X*mrk* has been proposed to control the migration of pigment progenitor cells from the neural crest and to inhibit certain cells lines from differentiating into macromelanophores (Adam *et al*., 1991; Weis and Schartl, 1998; Schartl, 2008).

Regulatory genes linked to the *Mdl* appear to control the type of melanic side-spotting pattern and the pattern's location on the fish's flank, as these features do not change with hybridization in *Xiphophorus* (Anders and Anders, 1978; Schartl, 1990). Non-linked regulatory genes appear to control the number of melanophore progenitor cells that differentiate into macromelanophores, the developmental timing of macromelanophore appearance, as well as the size and number of spots in *Xiphophorus* (Anders *et al*., 1973, 1984; Vielkind *et al*., 1976; Anders and Anders, 1978; Vielkind and

Vielkind, 1982; Gutbrod and Schartl, 1999). The identities of these regulatory genes are still unknown, but some studies have identified candidates for the *Xiphophorus* autosomal locus *R(Diff)*, named for "*regulatory* or *differentiation"*, that appears to control the terminal differentiation of melanophore progenitor cells into macromelanophores (Vielkind, 1976; Vielkind and Vielkind, 1982; Vielkind *et al*., 1982), and may have additional functions in pattern expression (Schartl, 2008). Although not yet investigated for the melanic side-spotting pattern, 22 candidates for melanoma regulation were identified as part of the *R(Diff)* region in *Xiphophorus* hybrids with the spotted-dorsal fin pattern (for a detailed description, see: Lu *et al*., 2017). Many of the identified genes have known functions that could affect pattern expression and other physiological or behavioral traits, including cell cycle regulation, cell differentiation, cell signaling, cell migration, cell structure and adhesion, and transcription and translation factors (Lu *et al*., 2017).

Few candidate genes have been identified that are linked to melanic side-spotting patterns, and their specific functions in patterning or in phenotypic correlations are still unknown. Since a variety of molecular systems can regulate both pigmentation and other traits (e.g., melanocortins, monoamines; see: *2.3 Mechanisms of Pigmentation-Phenotype Correlation*), we suggest that parallel research studies be conducted to identify each gene system's contribution to the pattern's expression and to its correlation with other traits. Non protein-coding mechanisms that affect gene expression and protein function should also be considered, including epigenetic regulation, gene promoters, non-coding RNAs, and post-translational modifications (Ducrest *et al*., 2008; Kottler and Schartl, 2018).

2.7.4 Temperature-Sensitivity Mechanism

Gene x environment interactions can affect presence and expression of the melanic side-spotting pattern (Vielkind and Vielkind, 1982). In a subset of *G. holbrooki* and *Poecilia latipinna* populations, the pattern is temperature-sensitive with reduced penetrance at higher temperatures (Schröder, 1964; Angus, 1983, 1989; Angus *et al.*, 1999; Horth, 2006). Temperature-sensitivity has not been identified in many populations or in other species, although expression of the spotted-dorsal fin pattern in *Xiphophorus* hybrids also exhibited incomplete penetrance at high water temperatures (Perlmutter and Potter, 1988). We propose a few evolutionary hypotheses for why temperature-sensitivity is limited to only a subset of populations within a species. First, the constitutively-expressed and temperature-sensitive melanic side-spotting patterns may have evolved independently within a species through different genes (Angus, 1989). Second, these populations share the same melanic side-spotting pattern locus but have different alleles, only some of which confer temperature-sensitivity (Angus, 1989). For example, a hypothetical protein important for macromelanophore determination might function constitutively in one population, but a mutation in that protein could have evolved in another population that makes its splice variation or tertiary structure temperature-sensitive. Finally, temperature-sensitivity could be an additional regulatory gene or locus that has evolved independently in a subset of populations.

Temperature-sensitivity appears to be genetically controlled, as it was inherited paternally in *G. holbrooki* (Horth, 2006). The molecular mechanism for temperature-sensitivity could be either in the *Mdl* or in regulatory loci. Molecular interactions, gene expression, and protein instability generally increase with temperature. *G. holbrooki* and

P. latipinna show an unusual trend in the opposite direction, similar to mammals that express a Himalayan pattern in which they are normally white but body areas exposed to low temperatures express melanin pigmentation; increases melanin synthesis via increased tyrosinase activity at low temperatures is a key mechanism for melanism in mammals (Kidson and Fabian, 1981; Kwon *et al*., 1989). Angus (1989) found tyrosinase activity in temperature-sensitive *P. latipinna* was elevated at higher temperatures when expression of the spotting pattern was weak; therefore, tyrosinase activity does not seem to be the temperature-sensitivity mechanism. In spotted-dorsal fin *Xiphophorus* hybrids, higher temperatures led to multi-vesiculated macromelanophores, which Perlmutter and Potter (1988) suggest is due to inhibition of melanin synthesis within vesicles. Alternatively, temperature-sensitivity could result from failure to transport tyrosinase to the melanosomes in these pigment cells (hence the empty vesicles), as retention of tyrosinase by the endoplasmic reticulum was higher at elevated temperatures in *himalayan* mouse cells (Halaban et al., 2000). Mutations that lead to increased stability of tertiary protein structure at low temperatures are another potential mechanism. Finally, we should consider that temperature-sensitivity may not be due to polymorphisms in protein-coding genes; for example, temperature-sensitive epigenetic regulation of pigmentation gene expression is possible. A shared temperature-sensitive molecular system could lead to the correlated expression of the melanic side-spotting pattern and other traits, as seen in a temperature-sensitive population of *G. holbrooki*. Males of the same genotype were exposed to different temperatures, so they were either unspotted or spotted, and these patterns correlated with behavioral differences (Horth, 2003).

2.8 Evolutionary Origins

Although melanic side-spotting patterns appear across Poeciliidae, the differences in inheritance and pattern features within and across species suggests that many of these patterns have different genetic mechanisms (Gordon, 1931b; Kallman and Atz, 1966). It is not clear at this time whether melanic side-spotting patterns arose through homologous, convergent, and/or parallel evolution within Poeciliidae, within specific genera, or even within species.

Here, we consider the possible evolutionary origins of melanic side-spotting patterns using the genus *Xiphophorus* as an example. First, pigmentation loci may have evolved independently in some species while others share a common ancestor. For example, the sex chromosomes of *X. variatus* and *X. maculatus* are homologous so they may share an ancestral spotted pattern, but the *X. helleri* autosome is not homologous with those two, and its spotted pattern may have evolved independently (Kallman and Atz, 1966; Schartl, 1990). Alternatively, the pattern genes were originally all on the same homologous chromosome and then translocated to another chromosome in a subset of species (Kottler and Schartl, 2018). Second, *Xiphophorus* may have a shared ancestral spotted pattern that was repeatedly lost in some species, which would explain why *Mdl* homologues exist in species without macromelanophore patterns (Weis and Schartl, 1998). Regulatory genes could have then evolved independently in different populations and species through parallel evolution, which would alter the development, expression, and location of the patterns (Anders *et al*., 1973; Vielkind and Vielkind, 1982; Schartl, 1990). *Mdl* on *Xiphophorus* sex chromosomes is linked to regions that may be hotspots for gene duplication, structural rearrangements, and recombination (Volff and Schartl, 2001), suggesting that a high incidence of parallel evolution is possible for some *Xiphophorus* species. Thus, a similar combination of homologous, convergent, and parallel evolution is hypothesized to explain the origin of multiple melanic side-spotting patterns in Poeciliidae.

2.9 Physiology

2.9.1 Androgens

Many endocrine and neuroendocrine substances are involved in the regulation of pigmentation, so physiological correlates are expected within morphs. Because the expression of the melanic side-spotting pattern is male-biased in some species (Angus, 1989; Horth, 2006), androgens have been hypothesized to affect the pattern's presence. Mature unspotted *G. holbrooki* females treated with methyl-testosterone developed male gonopodia but did not develop the spotted pattern (Angus, 1989; Horth, 2006). Although these results suggest that the pattern is sex-linked and not sex-limited in *G. holbrooki*, some caution should be applied. Methyl-testosterone is not a potent androgen in teleosts; if the pattern requires higher androgenic activity to appear in females, then 11-keto-testosterone is more likely to produce observable effects (Hishida and Kawamoto, 1970). Furthermore, secondary sexual characteristics have late life plasticity, but that does not necessarily mean that expression of the pattern also has late life plasticity. Expression of androgen receptors is often primed early in life, so testosterone may only induce pattern expression in females when applied before sexual maturity.

In species where both sexes express the melanic side-spotting pattern, males often have higher expression and heavier pigmentation (Gordon, 1927, 1951; Häussler, 1928; Bellamy, 1936; Mac Intyre, 1961; Borowsky, 1973; Angus *et al*., 1999; McDowall, 1999), so androgen levels are hypothesized to affect pattern variation among individuals and sexes, but circulating levels have not yet been measured in relation to initiation of spotting patterns. Individuals expressing melanin-based coloration often have higher circulating levels of sex steroids (rev: San-Jose and Roulin, 2018), but whether differences between spotted and unspotted morphs in poeciliids exist are still unknown. Hormones often have pleiotropic effects (Ketterson and Nolan, 1999), and high levels of sex steroids are associated with higher social dominance (rev: Oliveira *et al.*, 2002); thus, if higher pigmentation expression in males is due to higher levels of sex steroids, then it may represent a pleiotropic mechanism for the correlation of the melanic side-spotting pattern and behavior in poeciliids. Alternatively, sex steroid levels are correlated to behavior as opposed to the melanic side-spotting pattern, as suggested by the "challenge hypothesis" (Wingfield, 1984; Oliveira *et al.*, 2002). Briefly, spotted and unspotted morphs experience different social environments; if spotted morphs experience more aggressive social interactions, they might respond with higher circulating androgen levels.

2.9.2 Metabolism and Cortisol

Melanocortins were previously identified as a key candidate for pleiotropy between melanin-based color patterns and physiological traits. Melanocortins regulate melanin production, energy balance, and glucocorticoid release, among other traits (rev: Ducrest *et al.*, 2008; San-Jose and Roulin, 2018). Although spotted *G. holbrooki* males had higher basal cortisol levels than unspotted males, no significant difference in cortisol responses to an introduced stress (presence of a predator) was observed between morphs (Humphrey, 2019). This negative result may be a function of low sample size. To our knowledge, no study has measured other circulating hormones, such as melanocortins like α-melanocyte stimulating hormone. Metabolic rate also does not appear to correlate with the melanic side-spotting pattern in poeciliids, as spotted and unspotted morphs of *X. variatus* and *X. helleri* did not differ in metabolic rate (Meyer *et al*., 2006; Culumber, 2016). Spotted morphs might have a physiological compensation mechanism that counteracts changes in metabolic rate associated with pigmentation expression, or metabolic rate may not be a major outcome of melanocortin systems.

2.9.3 Melanoma

Macromelanophore-based patterns in Poeciliidae have been heavily studied as a model for skin cancer, particularly in hybrid *Xiphophorus* species; hybridization across populations or species creates a mismatch between the pigmentation pattern genes and their regulatory genes, which results in the unregulated differentiation of macromelanophores and the formation of tumors (rev: Anders *et al*., 1973, 1984; Anders and Anders, 1978; Vielkind and Vielkind, 1982; Schartl and Walter, 2016). However, melanomas have been observed in senescent individuals from natural populations of *X. variatus* and *X. nezahualcoyotl*, as well as a non-hybrid lab strain of *X. variatus* (Borowsky, 1973; Schartl *et al*., 1995; Fernandez and Bowser, 2008), so we will discuss the possible influence of melanomas on the evolution of melanic side-spotting patterns.

Melanoma is a potential mechanism for reducing the frequency of spotted individuals in a population (Gordon and Gordon, 1957). When malignant melanomas occur in hybrid *Xiphophorus*, they lower fish health and reduce survival, decreasing the fitness of spotted individuals; however, only a small proportion of these hybrid fish develop malignant melanomas, and melanomas are extremely rare in natural populations, so this disadvantage is not likely to be a general feature of this pattern (Häussler, 1928; Anders *et al*., 1973; Anders and Anders, 1978; Schartl, 2008); however, the rarity of melanomas in natural populations may also be a sampling artefact, as these fish are unlikely to survive and thus unlikely to be sampled. In *Xiphophorus*, only species with the constitutively active oncogene X*mrk* develop melanomas, so not all species with macromelanophore patterns are expected to experience the melanoma fitness disadvantage (Anders and Anders, 1978; Weis and Schartl, 1998). Although, other undiscovered mutations that lead to melanoma formation in macromelanophores could potentially still exist (Fernandez and Bowser, 2008). Finally, macromelanophore patterns can potentially evolve repeatedly through mutation; if individuals with these mutations also repeatedly develop melanomas, natural selection would act to eventually include regulatory genes that prevent this deleterious side-effect, which have been found in multiple populations and species of *Xiphophorus* (Gordon and Gordon, 1957; Atz, 1962; Borowsky, 1973). Thus, the fitness disadvantage associated with melanomas is likely not a strong selective pressure on the melanic side-spotting pattern (Anders *et al*., 1973; Schartl, 2008). Melanomas also often develop later in life in both non-hybrid and hybrid *Xiphophorus*, weakening selection against it; selection delayed is selection denied (Gordon and Gordon, 1957; Williams, 1957; Mac Intyre, 1961; Anders *et al*., 1973; Schartl *et al*., 1995). Selective pressures that act upon the melanic side-spotting pattern before melanoma development would thus have stronger effects on the pattern's frequency in populations.

The risk of developing malignant melanomas was higher for homozygotes of *X. maculatus* with the "fuliginosus" pattern and of *X. variatus* with the "punctatus-2" pattern, indicating that the melanoma fitness disadvantage may be stronger in homozygotes than heterozygotes (Mac Intyre, 1961; Borowsky, 1973). If the spotted pattern has other fitness advantages, these positive selective pressures on heterozygotes may outweigh the negative effect of melanomas on homozygotes (Borowsky, 1973; Kazianis and Borowsky, 1995), and thus the pattern would be maintained in the population as a polymorphism. Higher fitness of heterozygotes for the melanic side-spotting pattern could potentially explain the evolution of sex-linkage in some poeciliid species, as only one of the sex chromosomes carries the pigmentation allele, thereby preventing melanoma (Borowsky, 1973). Alternatively, because of their correlation with heavier expression of pigmentation patterns, melanomas may be under positive selection if expression is correlated with a fitness advantage. For example, *X. cortezi* females in some populations preferred males with heavier expression of a macromelanophore-based tail spot pattern (spotted caudal) that is associated with an increased risk of melanoma (Fernandez and Morris, 2008). Melanomas enhance the preferred pattern's appearance, which could lead to its maintenance through sexual selection in some populations (Fernandez and Morris, 2008). The possible fitness advantages of melanomas have not yet been studied in relation to melanic side-spotting patterns. Other aspects of fish health have not been investigated for the melanic side-spotting pattern. Melanin has known anti-parasitic properties in poeciliids, so spotted morphs could potentially have greater resistance to parasites (Meyer *et al.*, 2006; Horth *et al.*, 2013). Differences in the immune system exist in other species with melanin-based pigmentation patterns (rev: McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018), and thus should also be investigated for spotted and unspotted poeciliid morphs.

2.10 Conclusion

Melanin-based pigmentation patterns correlate with other traits across vertebrates through a variety of potential molecular and evolutionary mechanisms (rev: Gray and McKinnon, 2007; Ducrest *et al.*, 2008; Forsman *et al.*, 2008; McKinnon and Pierotti, 2010; Peiman and Robinson, 2017; San-Jose and Roulin, 2018). Poeciliids have proven a tractable model for studying pigmentation-phenotype correlations, so we have presented here the melanic side-spotting pattern as a model for investigating the mechanisms underlying the evolution of similar color patterns and their correlations with other traits across populations, species, and genera. Specific results are summarized in Table 2. Although the development and inheritance of melanic side-spotting patterns are well known, we still do not understand the genetics of the pattern or all aspects of the pigment cells' biology. Most of the research on correlated traits focuses on a subset of populations from *Xiphophorus* species and Eastern Mosquitofish (*G. holbrooki*), so we recommend extending research into more poeciliid populations, species, and genera to determine whether the results reviewed here are generalizable trends. Little is known about physiological correlates and life history traits of spotted morphs. Spotted males have altered social conflict behavior and social groups compared to unspotted males. The melanic side-spotting pattern is generally uncommon in populations despite being an evolutionarily stable phenotype, so multiple positive and negative selective pressures are presumed to act upon it and its correlated traits. Predation appears to be an important negative frequency-dependent selective pressure, but social interactions may also function in the frequency-dependent selection that maintains the spotted morph at a low population frequency. Physical environmental factors are likely also important, but only a couple studies on water conditions exist. It is unclear whether sexual selection acts upon the melanic side-spotting pattern, and it may depend on a variety of factors such as population and species, morph frequency, and social and physical environment. Finally, whether melanic side-spotting patterns arose through homologous, convergent, and/or parallel evolution within and across Poeciliid species remains to be determined.

2.11 Future Studies

Throughout this review, we identified what is still unknown within specific biological fields for the melanic side-spotting pattern in poeciliids. Here, we outline key research questions that may serve as a framework for future studies:

1. Which molecular mechanisms (sequential causation, co-expression, linkage disequilibrium, pleiotropy) and/or evolutionary mechanisms (correlational selection, co-adaptive selection, co-dependence, co-specialization) underlie the correlation between melanic side-spotting patterns and other phenotypic traits?
2. Which molecular signaling systems (e.g., peptide hormones, amino acid hormones, monoamines, sex steroids, neurotransmitters) affect the expression of the melanic side-spotting pattern, or are differentially expressed between morphs?
3. What gene(s) determine the presence or expression of the melanic side-spotting pattern to create the variation seen among populations and species? Do identified gene(s) pleiotropically affect or link to other molecular signaling systems that affect other phenotypic traits, such as physiology, life history, or behavior?
4. Does manipulation of molecular signaling systems that affect life history, physiology, or behavior also affect the presence or expression of the melanic side-spotting pattern, or vice versa?

5. Does the melanic side-spotting pattern or a correlated trait have an effect that is more disadvantageous in females than males that could lead to sexually antagonistic selection (and thus higher expression of the pattern in males)?
6. Are the correlated physiological, behavioral, and life history traits identified in this review a general trend across poeciliid populations, species, and genera? How do these correlated traits affect fitness of the melanic side-spotting pattern, especially survival and reproduction?
7. What social and physical environmental factors drive or break correlations in physiological, behavioral, or life history traits with melanic side-spotting patterns?
8. How do social and physical environmental factors interact with the pattern and its correlated traits to affect morph fitness? How do these interactions affect the maintenance and frequency of the melanic side-spotting pattern in populations and species?
9. What is the evolutionary origin of the polymorphic melanic side-spotting pattern? Did these similar patterns arise through homologous, convergent, and/or parallel evolution within and across species?

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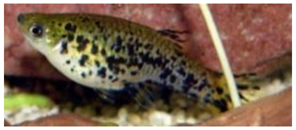
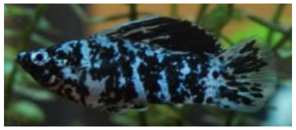
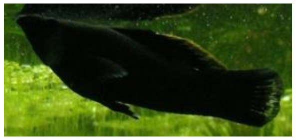
2.13 Tables

Table 1. Summary of the specific poeciliid pigmentation patterns included under the “melanic spotting pattern” definition of this review. Patterns are listed by species with name of the pattern in published literature, a brief description and representative photo, whether the pattern exists in the wild, the sex that the it primarily occurs in, and the mode of genetic inheritance.

Species	**Pattern Name**	**Representative Photo**	**Pattern Description**	**Wild or Lab?**	**Sex**	**Inheritance**	**References**
Gambusia holbrooki	spotted, mottled, melanistic, melanic	 Photo by Tanja Zerulla	small to large blotches on flanks, head, and fins	wild	male	Y chromosome	Myers, 1925; Regan, 1961; Angus, 1989; Horth, 2006; Kottler *et al*., 2020
Limia vittata	spotted	 Photo by T.M. Rodriguez-Cabrella	small to large blotches on flanks	lab	both	unknown	Haskins *et al*., 1960
Phalloceros caudimacultus	spotted, melanistic, melanic, mottled, blotched	 Photo licensed under a Creative Commons agreement[1]. Image has been modified to focus on the fish.	small to large blotches on the flanks, head, and fins	wild	both	autosomal	Myers, 1925; Trendall and Johnson, 1981; McDowall, 1999; Gutiérrez and García, 2007, 2011; McNeil and Wilson, 2008
Poecilia. formosa	spotted	Refer to photo for spotted *P. latipinna*	small to large blotches on the flanks, head, and fins	lab	clonal female that develops as male	paternal microchromosomes	Haskins *et al*., 1960; Schultz and Kallman, 1968; Turner *et al*., 1980; Schartl *et al*., 1997; Lampert *et al*., 2007; Nanda *et al*., 2007; Lamatsch *et al*., 2010, 2011
	black	Refer to photo for black *P. latipinna*	extreme form of spotting in which spots coalesce to cover almost or all of the body				

Poecilia latipinna	spotted, mottled, melanistic, melanic	 Photos licensed under a Creative Commons agreement. Image has been modified to focus on the fish and flipped horizontally.	small to large blotches on the flanks, head, and fins	wild	both	autosomal (single gene)	Schröder, 1964; Angus, 1983; Angus *et al*., 1999
	black	 Photos licensed under a Creative Commons agreement. Image has been modified to focus on the fish and flipped horizontally.	extreme form of spotting in which spots coalesce to cover almost or all of the body	lab	both	autosomal (single gene)	Schröder, 1964
Poecilia mexicana	spotted	Refer to photo for spotted *P. latipinna*	small to large blotches on the flanks, head, and fins	wild	both	autosomal	Schröder, 1964; Menzel and Darnell, 1973; Culumber *et al*., 2014
	black	Refer to photo for black *P. latipinna*	extreme form of spotting in which spots coalesce to cover almost or all of the body	lab			
Poecilia sphenops	spotted	Refer to photo for spotted *P. latipinna*	small to large blotches on the flanks, head, and fins	wild	both	autosomal (at least two independently segregating genes)	Myers, 1925; Schröder, 1964
	black	Refer to photo for black *P. latipinna*	extreme form of spotting in which spots coalesce to cover almost or all of the body	lab			
Poecilia velifera	spotted	Refer to photo for spotted *P. latipinna*	small to large blotches on the flanks, head, and fins	lab	both	unknown	Schröder, 1964
	black	Refer to photo for black *P. latipinna*	extreme form of spotting in which				

			spots coalesce to cover almost or all of the body				
Xiphophorus birchmanni	carbomaculatus	 *X. birchmanni* (cam) [2]	very few, large blotches on dorsoposterior of flank	wild	both	autosomal?	Rauchenberger *et al*., 1990; Walter *et al*., 2006
X. cortezi	atromaculatus, spotted	Photo not available	many spots that later fuse into blotches similar to carbomaculatus; located on dorsoposterior of flank and dorsal fin	wild	both	autosomal	Atz, 1962; Zander, 1965, 1967; Kallman, 1971; Rauchenberger *et al*., 1990
	carbomaculatus, spotted	 *X. cortezi* (cam) [2]	very few, large blotches on dorsoposterior of flank	wild	both	autosomal (single gene)	Atz, 1962; Zander, 1965; Kallman, 1971; Rauchenberger *et al*., 1990; Walter *et al*., 2006
X. couchianus	weakly spotted	Photo not available [3]	weakly spotted	wild (extinct)	both	unknown	Myers, 1925; Gordon, 1943
X. evelynae	spotted, speckled-1, speckled-2	 *X. evelynae* (eve) with speckled-2 [2]	punctate to small spots on dorsoposterior of flank	wild	both	unknown	Weis and Schartl, 1998; Walter *et al*., 2006
X. helleri	spotted, dabbed-1	 *X. helleri* (BxII) [2]	few punctate to small spots on flank	wild	both	autosomal (single gene)	Atz, 1962; Kallman and Atz, 1966; Kallman, 1975; Franck *et al*., 1998; Weis and Schartl, 1998; Walter *et al*., 2006

X. maculatus	pulchra, spotted	 *X. maculatus* (Up-2) with spotted pattern-4 [2]	punctate spots to large blotches on flank	wild	both	X chromosome	Bellamy, 1924, 1936; Gordon, 1927, 1931a, 1937, 1943, 1946, 1951; Fraser and Gordon, 1929; Atz, 1962; Anders *et al*., 1973; Walter *et al*., 2006
	fuliginosus, fury	 Figure A from Mac Intyre (1961)	undefined spots that appear diffusely on flank and fins to give a “sooty” look	lab	both	Y chromosome	Koßwig, 1938; Mac Intyre, 1961
	nigra	 *X. maculatus* (nigra) [2]	a few large blotches coalesce into a black band or “smear” on the flank	wild	both	X chromosome	Bellamy, 1924; Gordon, 1927, 1931a, 1931b; Atz, 1962; Kallman and Borkoski, 1978; Walter et al., 2006
	black	 Photos licensed under a Creative Commons agreement. Image has been modified to focus on the fish and flipped horizontally.	extreme form of spotting in which spots coalesce to cover almost or all of the body	lab	both	unknown	Gordon, 1927
X. meyeri	Spotted [4]	 *X. meyeri* (meyeri) [2]	punctate to medium spots on flank	wild	both	autosomal	Schartl and Schröder, 1987; Weis and Schartl, 1998; Walter *et al*., 2006

X. montezumae	spotted	 *X. montezumae* (Rascon) [2]	small punctate spots on flank by the mid-lateral line, but occasionally also on dorsal fin	wild	both	autosomal	Gordon, 1943; Kallman and Atz, 1966; Zander, 1967; Kallman, 1983; Rauchenberger *et al*., 1990; Weis and Schartl, 1998; Walter *et al*., 2006
	marmoratus	Photo not available	large blotches on flank located ventrally of mid-lateral line	wild	both	unknown	Weis and Schartl, 1998
X. nezahualcoyotl	spotted	 *X. nezhualcoyotl* (El Salto) [2]	small to medium spots on flanks	wild	both	X chromosome and Y chromosome	Kallman, 1983; Rauchenberger *et al*., 1990; Weis and Schartl, 1998; Walter *et al*., 2006; Fernandez and Bowser, 2008
X. variatus	punctatus (1-3), spotted, blotched, speckled	 *X. variatus* (Zarco) with punctatus-2 [2]	punctate to large blotches depending on the punctatus pattern (2 is heavier than 1) that are restricted to on or above mid-lateral line on flank	wild	both	X chromosome and Y chromosome	Gordon, 1943; Atz, 1962; Kallman and Atz, 1966; Borowsky, 1973; Kazianis and Borowsky, 1995; Schartl *et al*., 1995; Walter *et al*., 2006
X. xiphidium	spots, spotting, flecked	 *X. xiphidium* (RP) [2]	punctate to small blotches restricted to on or above mid-lateral line on flank	wild	both	X chromosome	Gordon, 1943; Atz, 1962; Kallman and Atz, 1966; Walter *et al*., 2006

Notes: 1. Creative Commons photos licensed under the following agreement: https://creativecommons.org/licenses/by-sa/3.0/legalcode
2. Images provided by the *Xiphophorus* Stock Center at Texas State University (Walter *et al*., 2006).
3. The spotted pattern has not been observed in *X. couchianus* since 1943. This species is extinct in the wild (Walter *et al*., 2006).
4. *X. meyeri* also exhibits a spotted pattern. The cell types have been considered intermediate between micro- and macromelanophores (Schartl and Schröder, 1987; Weis and Schartl, 1998) and as macromelanophores (Walter *et al*., 2006), so it is tentatively included in this review.

Table 2. Summary of traits and selective pressures investigated for melanic spotting patterns in poeciliid species. We briefly describe the experiment and results. Direction of effect is listed as increased (+), decreased (–), and no difference (/) for the spotted morph compared to the unspotted morph.

Trait	**Test**	**Result or Effect**	**Species**	**Reference**
Molecular				
Macromelanophore-determining locus	Candidate genes linked to spotting pattern *Mdl*	*GIPC1*	*Gambusia holbrooki*	Kottler *et al*., 2020
Macromelanophores	Microscopy	Larger size, thicker and more dendritic arms, more melanosomes	*Gambusia holbrooki* *Poecilia sphenops* *Xiphophorus* hybrids *Xiphophorus maculatus*	Gordon 1931a; Regan, 1961; Vielkind, 1976; Anders *et al*., 1984; Kottler *et al*., 2020
	Skin regeneration and grafts	Inhibit differentiation and induce destruction of micromelanophores	*Xiphophorus* hybrids	Schmidt, 1978
	Adrenaline application to stimulate melanosome aggregation	No aggregation	*Gambusia holbrooki*	Kottler *et al*., 2020
Temperature-sensitivity mechanism	Tyrosinase activity	Activity increased with increasing temperatures; not the mechanism	*Poecilia latipinna*	Angus *et al*., 1999
Development				
	Crosses to determine genetic inheritance of spotting pattern Observations in the wild and in the lab	Born unspotted; pattern fully expressed after sexual maturity	*Gambusia holbrooki* *Phalloceros caudimaculatus* *Poecilia latipinna* *Poecilia sphenops* *Xiphophorus* hybrids *Xiphophorus helleri*	Bellamy, 1924, 1928; Myers, 1925; Gordon, 1931b; Schröder, 1964; Angus, 1983; Martin, 1984; Gutiérrez and García, 2007
		Male expression > female expression	*Phalloceros caudimaculatus* *Poecilia latipinna* *Xiphophorus* hybrids *Xiphophorus cortezi* *Xiphophorus maculatus* *Xiphophorus variatus*	Gordon, 1927, 1951; Häussler, 1928; Bellamy, 1936; Mac Intyre, 1961; Atz, 1962; Borowsky, 1973; Angus, 1983; McDowall, 1999
		Expression increases with age	*Gambusia holbrooki* *Poecilia latipinna* *Xiphophorus cortezi* *Xiphophorus helleri* *Xiphophorus variatus*	Myers, 1925; Atz, 1962; Kallman and Atz, 1966; Kallman, 1971; Angus, 1983; Angus *et al*., 1999; Meyer *et al*., 2006

Physiology				
Androgens	Methyl-testosterone on expression of pattern in adult females	/	*Gambusia holbrooki*	Angus, 1989; Horth, 2006
Glucocorticoids	Basal cortisol levels	+	*Gambusia holbrooki*	Humphrey, 2019
	Cortisol response to predator	/		Humphrey, 2019
Routine metabolic rate	Oxygen consumption rate in a respirometer	/	*Xiphophorus helleri*	Meyer *et al*., 2006
		/	*Xiphophorus variatus*	Culumber, 2016
Stress resistance	Activity during confinement test	+	*Xiphophorus variatus*	Culumber, 2016
Melanoma	Development in homozygotes compared to heterozygotes	+	*Xiphophorus maculatus*	Koßwig, 1938; Mac Intyre, 1961
		+	*Xiphophorus variatus*	Borowsky, 1973
Behavior				
Feeding	Juveniles raised in presence of phenotypically similar or different adults	/	*Gambusia holbrooki*	Culumber *et al*., 2018
Antipredator	Response to predator odorants	/	*Gambusia holbrooki*	Kraft, 2016
	Freezing behavior	/		Humphrey, 2019
Dominance	Altered frequency of spotted morphs & density	+	*Gambusia holbrooki*	Martin, 1977
	Juveniles raised in presence of phenotypically similar or different adults	+		Kraft *et al*., 2018
	Paired male conflicts	/	*Xiphophorus helleri*	Franck *et al*., 2001
Aggression	Temperature-sensitive siblings in social group (same genotype, different phenotype)	/	*Gambusia holbrooki*	Horth, 2003
	Juveniles raised in presence of phenotypically similar or different adults	/		Kraft *et al*., 2018
Behavioral flexibility	Juveniles raised in presence of phenotypically similar or different adults	–	*Gambusia holbrooki*	Kraft *et al*., 2018
Boldness	Novel environment	–	*Xiphophorus variatus*	Culumber, 2016
Sexual activity	Altered frequency of spotted morphs & density	+ at low spotted frequency & high density – at equal frequencies	*Gambusia holbrooki*	Martin, 1977
	Female paired with both morphs with and without barriers	≠		Nelson and Planes, 1993
	Observed sexual behavior in the wild	/ or + depending on behavior		Karplus and Algom, 1996
	Temperature-sensitive siblings in social group (same genotype, different phenotype)	+		Horth, 2003
	Juveniles raised in presence of phenotypically similar or different adults	–		Kraft *et al*., 2018
	Social groups before and after predator exposure	+		Humphrey, 2019

	Morphs with different social experience (low or high frequency of spotted morph) grouped with females	+		Culumber *et al*., 2020
Female preference	Altered frequency of spotted morphs & density	–	*Gambusia holbrooki*	Martin, 1977
	Female had visual cues of both morphs	/		Martin, 1986
	Female paired with both morphs	/ with barriers - without barriers		Nelson and Planes, 1993
	Observed sexual behavior in the wild	+		Karplus and Algom, 1996
	Female had visual cues of both morphs	–		Taylor *et al*., 1996
	Two-model choice tests	+		Gould *et al*., 1999
	Females from different populations (spotted absent vs. spotted present) in 3-chamber choice test	– unspotted females + spotted females		Bisazza and Pilastro, 2000
	Females in 3-chamber choice test	–		Bisazza *et al*., 2001
	Temperature-sensitive siblings in social group (same genotype, different phenotype)	/		Horth, 2003
	Dichotomous choice test	– unspotted females from non-sulfidic spring / unspotted females from sulfidic spring + spotted females from sulfidic spring	*Poecilia mexicana*	Culumber *et al*., 2014
	School and dichotomous choice tests in clear conditions	– (schools) & / (males) unspotted females + spotted females	*Xiphophorus helleri*	Franck *et al*., 2001
Social group	Observations of social interactions in lab and wild	More social partners & higher association with females over males	*Gambusia holbrooki*	Kraft *et al*., 2016
	School choice test	Preferred phenotypically similar school	*Poecilia* hybrids	McRobert and Bradner, 1998; Bradner and McRobert, 2001
	School choice test based on experience	Preferred to school with familiar color pattern		Ledesma and McRobert, 2008
Life History Traits				
Gestation	Length of gestation for different female and male morphs	/	*Poecilia sphenops*	Schröder, 1964
Brood sex ratio	Crosses to determine genetic inheritance of spotting pattern	Usually equal sex ratios, although some male-biased	*Gambusia holbrooki*	Horth, 2006
Survival	Mesocosms with varying frequency	+ at low spotted frequency / at high spotted frequency	*Gambusia holbrooki*	Horth and Travis, 2002
	Field survival	/		Horth, 2004
	Crosses to determine genetic inheritance of spotting pattern	– –	*X. maculatus* *Xiphophorus* hybrids	Mac Intyre, 1961 Koßwig, 1927

Body size	Measured in nature and in the lab	+	*Gambusia holbrooki*	Martin, 1977; Horth *et al*., 2010
	Temperature-sensitive siblings (same genotype, different phenotype)	/		Horth, 2003
	Measured for same-age morphs in the lab	/	*Xiphoporus helleri*	Meyer *et al*., 2006
Body condition	Juveniles raised in presence of phenotypically similar or different adults	+	*Gambusia holbrooki*	Culumber *et al*., 2018
Environment				
Predation	Consumption by predator	–	*Gambusia holbrooki*	Martin, 1977; Bonner, 1980 in: Martin, 1986
		– at low spotted frequency / at high spotted frequency		Horth, 2004
		/		Humphrey, 2019
		–	*Xiphophorus helleri*	Dürr, 1996 and Becker, 1997 in: Franck *et al*., 2001
	Hunting activities by predator	/	*Gambusia holbrooki*	Humphrey, 2019
		–	*Xiphophorus helleri*	Dürr, 1996 and Becker, 1997 in: Franck *et al*., 2001
Sulfidic springs	Dichotomous choice test (females from sulfidic spring)	/ unspotted females + spotted females	*Poecilia mexicana*	Culumber *et al*., 2014
Turbidity	School choice experiments in turbid conditions compared to clear conditions	+	*Xiphophorus helleri*	Franck *et al*., 2001
Temperature	Simulated population model	Seasonal changes around an evolutionarily stable mean	*Gambusia holbrooki*	Horth and Panayotova, 2012
	Simulated population model in response to climate change	–		Panayotova and Horth, 2018
	Penetrance of pattern expression	Higher in winter temperatures	*Gambusia holbrooki*	Angus, 1989; Horth, 2006
			Poecilia latipinna	Schröder, 1964; Angus, 1983; Angus *et al*., 1999

CHAPTER 3

Social Behavior of Male Eastern Mosquitofish (*Gambusia holbrooki*) Color Morphs Depends on Social Context

3.1 Abstract

Male Eastern Mosquitofish (*Gambusia holbrooki*) exhibit a discrete color polymorphism. Most males are silver but a low frequency are mottled with black and brown blotches. Color patterns can correlate with other traits like behavior within individuals, resulting in alternative phenotypes or morphs. Previous studies present conflicting results on whether and how male mosquitofish morphs differ in social behavior. To resolve these conflicts, I first standardize behavioral measurement by creating a comprehensive ethogram for male mosquitofish. Most studies used mix-sex groups, but I also observed males in the field interacting in groups of only males or only females, which have not yet been studied systematically. This study explores how the expression of social behavior differs between the two male morphs in three different social contexts: (1) with a group of females, (2) in a male-male dyad, and (3) in a mix-sex group. Overall, the expression of social behavior depends on both focal morph and the phenotypes of social partners. When interacting only with females, mottled males are more assertive than silver males; however, mottled males lower their high assertive attention towards females to match the levels of silver males in mix-sex groups. Both morphs behave differently in mix-sex groups compared to female-only groups. Furthermore, when a mottled male is present, silver males behave differently towards females than they do around only silver males. In both dyads and mix-sex groups, mottled males dominate silver males. Neither morph appears to be inherently more aggressive, as the dominance advantage of mottled males derives from

silver males acting more submissive around mottled males. Both morphs alter their use of specific behaviors and of transitions between behaviors based on the phenotype of the interacting individual and the social context. In conclusion, variation in social environment affects the expression of social behavior differently for each male mosquitofish morph, and its effect on the maintenance of mottled morphs at low frequencies in mosquitofish populations should be further investigated.

3.2 Introduction

Color patterns have been observed to correlate with behavioral traits across vertebrates, resulting in alternative phenotypes or alternative morphs (rev: Brockmann, 2001; Ducrest *et al.*, 2008; McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018). Behavioral differences may affect the fitness and selection of these morphs, leading to differences in their frequency within populations (rev: Brockmann, 2001; Ducrest *et al.*, 2008; McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018). If color pattern is genetically determined, correlated differences in behavioral expression between morphs could be due to direct genetic effects (rev: Moore *et al.*, 1997; Wolf *et al.*, 1998; McGlothlin *et al.*, 2010; Saltz, 2017). Given that multiple morphs exist in a population, expressed social behaviors may also be dependent on the indirect genetic effect of the interacting individual's phenotype (i.e., gene x environment interactions; rev: Moore *et al.*, 1997; Wolf *et al.*, 1998; McGlothlin *et al.*, 2010; Saltz, 2017). Variation in social environment could affect the expression of social behavior by color morphs.

Color patterns are associated with socially mediated behavioral differences across vertebrate taxa. In Harris's sparrows (*Zonotrichia querula*), larger black badges signal dominant social status (Rohwer and Ewald, 1981). Dominant males displace subordinate males to increase their access to food caches; however, aggression not related to feeding is instead focused on birds of similar rank (Rohwer and Ewald, 1981). For the tawny dragon lizard (*Ctenophorus decresii*), orange and gray morphs use the same behavioral pattern regardless of competitor, but yellow morphs change their strategy based on competitor morph (Yewers *et al.*, 2016). Territorial males of the cichlid *Astatotilapia burtoni* also alter their behavior based on competitor; both morphs initiate more agonistic encounters with a competitor of the opposite color, although yellow morphs still ultimately dominate over blue morphs (Korzan and Fernald, 2007). Therefore, expression of social behavior is known to depend on both social context and the phenotypes of conspecific individuals across taxa.

Eastern Mosquitofish (*Gambusia holbrooki*) males exhibit a genetically-determined, discrete color polymorphism in which most fish are unspotted (silver), but a low frequency of individuals are unpigmented with black and brown blotches on their bodies (mottled; first described by Myers, 1925; Regan, 1961; Angus, 1989; Horth, 2006; Zerulla and Stoddard, 2021). Whether morphs differ in sexual activity is unclear, as previous studies offer conflicting results (Martin, 1977; Nelson and Planes, 1993; Karplus and Algom, 1996; Kraft *et al.*, 2018; Culumber *et al.*, 2020). These disparities could derive from differences in experimental design, but also potentially from lack of standardization in behavioral analysis, as each study used slightly different subsets of male mosquitofish behaviors. To resolve these issues, I define an ethogram of male mosquitofish social behaviors to create a standardized description for this species. I then use this ethogram to comprehensively score male mosquitofish morph behavior, which allows for the evaluation of differences in overall behavior as well as specific changes in behavior use. In the presence of females, mottled males direct more aggression towards silver males than they receive in return, as silver males focus their aggression on other silver males (Martin, 1977; Horth, 2003), indicating that morphs alter their behavior based on the interacting male's phenotype. Mottled juveniles are not inherently more aggressive than silver juveniles, but silver juveniles adjust their behavior towards adult males and females in response to social context more than mottled juveniles (Kraft *et al.*, 2018). Differences in mosquitofish morph behavior seem to be dependent on social context – in these cases, the sex and morph of interacting social partners.

The robustness of behavioral studies depends on the replication of the social environment experienced by animals in the wild (Travis, 2020). Kraft *et al.* (2016) previously found that mottled males interact with females more than silver males in the wild, suggesting that morphs experience different social environments. I first observed mosquitofish in the field to define the types of social environments experienced by males. Previous studies used mix-sex groups with multiple males and females, but I also observed males in the field interacting only with males or only with females; these contexts have not yet been studied systematically. Therefore, I investigate how the expression of intra- and inter-sexual social behavior differs between adult male mosquitofish morphs across different social contexts: (1) with a group of females, (2) in a male-male dyad, and (3) in a mix-sex group.

3.3 Methods

3.3.1 Field Observations

Eastern Mosquitofish (*G. holbrooki*) males were observed at Hennington Pond (HP: 25.759848°N, 80.376975°W) on the MMC campus of Florida International University

(FIU) in Miami, Florida. Three independent observers (Tanja Zerulla, Jessica Vallori, and Marina Salvador) went to the pond from 23-30 Oct 2017 to observe the social interactions of male mosquitofish. Observations (n=120) were conducted both in the morning and afternoon on clear, overcast, and rainy days with ambient air temperatures ranging from 22-30°C. Observers used a 0.25-meter x 0.25-meter PVC square set on the substrate of the pond to define an observation area. Adult *Gambusia* species prefer to swim in shallow, open areas during the day (Pyke, 2005), so observers set up the square in areas with <30 cm of water and low vegetation or no vegetation. Locations were distributed haphazardly along the pond's edge.

After set-up, observers waited for mosquitofish to enter the area again and interact with each other for one minute. An adult male was then chosen haphazardly and observed to determine the type of social partners with which they typically interact. Observations lasted for a minimum of 30 s and continued until the focal male left the square. A social interaction was defined as when the focal male and another mosquitofish were within approximately a body length of each other. Social partners were noted as mix-sex, only males, only females, or none. Adult mosquitofish were identified by their large size compared to juveniles, while females were distinguished from males by their swollen, gravid sides (Pyke, 2005). When possible, observers also used two additional identifying features between the sexes: the large black periproctal spot present just in front of the gonopore in females, and the gonopodium, a modified anal fin used by males to transfer sperm during mating (Pyke, 2005). Social groups observed in the field were used to determine the social contexts for laboratory experiments. Based on field observations of social groups (range: 1 to 14 individuals), mosquitofish males swam alone (4%), with only females (21%), with only males (13%), and in mix-sex groups (62%). Males interacted with groups composed of only the same-color morph, mixed-color morphs, or different-color morphs. (Morph percentages are unavailable, as observations of male color were originally only noted as verbal correspondences among observers.)

On 13 Nov 2017, I conducted 16 additional observations to determine the number of mosquitofish normally present in an area. The area captured by the PVC square approximates the size of a 20 L aquarium (40 cm long x 25 cm high x 21 cm deep). I recorded the minimum and maximum number of individuals present over a 1-min interval to determine group sizes for laboratory behavioral observations (range: 1–14). Based on the mean of maximum observed density (356.7 individuals/m^3), the behavioral arena can house a maximum of seven fish. In this study, the largest tested social group is five individuals to reduce stress on the fish that may occur at the maximum density.

3.3.2 Behavior Experiment

G. holbrooki were collected by dip-netting from three different locations on the MMC campus of FIU: Hennington Pond (HP: 25.759848°N, 80.376975°W), Owa Ehon Pond (OE: 25.758544°N, 80.373335°W), and Student Housing Pond (SHP: 25.757011°N, 80.370483°W). Additional wild fish were collected from Tamiami Canal near the Everglades (TC: 25.7619835°N, 80.5024283 °W) and from a private pond (PP: 25.707068°N, 80.299002°W). Fish were housed in mix-sex and mixed-color groups in 38-liter aquaria with crushed limestone pea gravel and PVC shelters at 22°C with a 12:12 light cycle. Fish were fed TetraColor Tropical Flakes (Tetra) daily *ad libitum*. Experimental fish were moved to separate 38-liter aquaria 24 h before testing. All field and lab methods were carried out under the approval of FIU's Institutional Animal Care and Use Committee (IACUC-15-027-CR02; IACUC-16-012-CR01).

The behavioral arena consisted of an 19 L aquarium (40 cm long x 25 cm high x 21 cm deep) with limestone substrate on the bottom. Two fluorescent lights were positioned on either side of the aquarium to illuminate it. This illumination caused reflection on the aquarium's sides, so white mesh netting was placed on the sides to prevent fish from seeing and interacting with their own reflection. A white foam board was placed on the back of the aquarium to provide contrast for observing the fish. A video camera (GoPro Hero4, 1080p, 60 fps) was placed on a stand in front of the aquarium. After recording began, the entire set-up was covered with black cloth on all sides to prevent disturbance to the fish's behavior.

Fish acclimated in 2-liter plastic aquaria for 5 min prior to testing. All fish were then introduced at the same time to the aquaria to prevent time-based dominance, although mosquitofish are not known to exhibit territoriality (Itzkowitz, 1971). Based on pilot recordings of social behavior and of individual fish, both mottled and silver males recover natural behavior within a minute of introduction to the aquarium (median recovery time = 12.9 s, IQR = 19.2 s; maximum recovery time = 43.2 s). I recorded no significant difference in recovery time to normal behavior between silver and mottled males (Mann-Whitney U Test: P = 0.15; n = 8 per morph). Silver males in the field exhibited a median number of 6.6 social interactions per minute. To observe approximately 100 social interactions for further behavioral analysis, mosquitofish should be observed for 15 mi; behavior was recorded for 30 min to cover the variation.

To investigate behavior in different social contexts, I tested 8 silver and 8 mottled males in three social settings: (1) with a male opponent only, (2) with a group of females, and (3) with both a male opponent and a group of females. A pair of mottled and silver males that were similar in size were tested together in all contexts on the same day. Water was changed in between behavioral tests to prevent any residual effect of odorants or other released chemicals on the next trial. All fish were given 1 h between tests to recover from possible stress. Order of trials was randomized each day. At first, experiments focused on interactions of silver males with both silver and mottled male opponents, but recorded mottled males only with silver male opponents, as I had not observed mottled males interacting together during the field observations; however, I observed pairs of mottled males interacting together a few months later while collecting fish. An additional 8 mottled males were tested later with a mottled male opponent and with a mottled male opponent and a group of females.

3.3.3 Length and Melanism Analysis

Fish length was estimated from images captured from the video recording using *ImageJ* software (v.1.52k; Schneider *et al.*, 2012). The scale was calibrated using the known distances in the aquarium. For each fish, the standard length (tip of the snout to the end of the caudal peduncle) was measured twice and then averaged. The video-based measurements taken in the behavioral arena were corrected based on 127 measurements comparing this protocol and their caliper measurements: 0.9348 * mean video-based length + 1.103 mm. To calculate the proportion of black spots present on the body of mottled males and of periproctal spots on females, images from both sides of the fish were taken from the video, the fish's body was outlined in Adobe *Fresco* (2022), and all black spots were traced and filled in and exported to *ImageJ* which made the calculation.

3.3.4 Behavioral Analysis

Before scoring, the behavioral actions of male mosquitofish needed to be defined in an ethogram. Two observers, both I and an assistant with no previous knowledge of fish behavior (Jessica Vallori), independently watched the recorded videos. We identified and defined any unique behaviors that we observed, then came together to create a consensus on the ethogram. Names and descriptions were matched to previously defined behaviors for mosquitofish whenever possible (Itzkowitz, 1971; Peden, 1972). An effort was taken to name behaviors that encompassed a distinguishable action or combination of actions. For example, lateral display is described as two fish in parallel, usually with fin-spreading and gonopodial extension. This behavior complex could be broken into three different actions that overlap in time, but together they form a recognizable and repeated complex, and thus were defined as a single behavior. Behaviors grouped into broader categories: mating, assertive attention, and submission. Focal male behavior was then scored in BORIS (v.7.10.2; Friard and Gamba, 2016) for the full 30 min videos using the behaviors defined in this ethogram. Both the frequency and duration of individual behaviors were recorded for state events, while only frequency was recorded for point events.

3.3.5 Statistical Analysis

Previous studies of behavior in mosquitofish primarily examined frequency of assertive behaviors as opposed to duration (e.g., Horth, 2003). However, while defining behaviors in the ethogram, I observed that some fish exhibited a high frequency of actions over a short period, while others exhibited a low frequency of actions over a long period. I hypothesized that duration would be a better indicator of assertive attention than frequency, so the total frequency of assertive behaviors and the total duration of assertive attention were compared with Spearman's rank correlation test. Additionally, many studies only examine behaviors in which fish actively approach, follow, or chase another fish. I compared the duration of these active assertive behaviors (approach, follow, chase, circling) with the total duration of assertive attention using Spearman's rank correlation test to ensure that my results were comparable with previous studies. I also wanted to determine whether scan-sampling would be a viable method to reduce time needed to score behavior for future studies. In this case, I extracted the duration of assertive attention for 1-min in every 3-min interval, resulting in 10 scan samples per trial. These values were then binned and compared against the total duration of assertive attention using a Spearman's rank correlation test. I determined that total frequency of assertive behaviors ($\rho = 0.86$, $P<0.001$), duration of active assertive behaviors ($\rho = 0.77$, $P<0.001$), and scan-sampled duration of assertive behaviors ($\rho = 0.98$, $P<0.001$) are all correlated with the total duration of assertive behaviors (Fig. S1).

To determine whether mottled and silver males exhibit different behaviors across social contexts, I used Bayesian mixed effects models in which the focal male morph as well as the sex and morph of stimulus and observer fish were included as fixed effects. Because focal males were repeated across contexts, subject was included as a random effect. To create a value that encompassed both assertive attention and submission, dominance index was calculated as: [duration of focal male assertive attention] / [duration of focal male assertive attention + duration of focal male submission]. Assertive attention was modelled using a beta regression distribution. Zero-inflated beta regression models failed to converge for submission and dominance index, so 0.001 was added or subtracted to each variable, respectively, before running the models. Using the posterior predicted probability distribution, contrasts were calculated using median marginal estimates and highest posterior probabilities density (HPD) for the 0.95 credible intervals, with a multivariate *t*-distribution adjustment. Modelling and contrasts were performed with the R packages: rstanarm v.2.21.3, brms v.2.19.0, tidybayes v.3.0.4, and emmeans v.1.8.5 (Bürkner, 2021; Goodrich *et al*., 2022; Lenth, 2022; Kay, 2023). Though I wanted to investigate whether behavior depended on proportion of melanism for mottled males, I found that body length and melanism were highly correlated ($\rho = 0.70$, $P<0.001$) and thus confounded, so I did not continue forward with that analysis (Fig. S2-S3).

I performed a principal components analysis (PCA) on the frequency and duration of each identified behavior to evaluate their contribution to male mosquitofish social behavior using the R packages tidyverse v.2.0.0, factoextra v.1.0.7, and ggfortify v.0.4.16 (Tang *et al*., 2016; Wickham *et al*., 2019; Kassambara and Mundt, 2020). To investigate how morphs may use different specific behaviors depending on social context, I used each of the identified principal components (PC) as responding variables in Bayesian mixed effects models. Focal male morph as well as the sex and morph of stimulus and observer fish were included as fixed effects, while subject was included as a random effect. Models used skewed normal distributions. Using the posterior predicted probability distribution, contrasts were calculated using median marginal estimates and HPD for the 0.95 credible intervals, with a multivariate *t*-distribution adjustment.

I analyzed behavioral transitions (e.g., A → B, in which behavior A is followed by behavior B) following a sequential analysis previously described by Egge *et al.* (2011) and Worthington and Swallow (2011). Briefly, I calculated counts of first order behavioral transitions for each trial. These counts were then summed within social context and used to calculate transition frequencies. Behavioral transitions that did not occur (i.e., frequency = 0) or that transitioned with "non-social" or "exclude" were removed from subsequent analysis. I then calculated modified z-scores based on median absolute deviation and associated Bonferroni-adjusted P-values for the remaining transitions to determine which behavioral transitions significantly contributed to morph behavior in each context. Significantly contributing behavioral transitions with frequencies above 0.01 were presented in kinematic diagrams. Frequencies of these significantly contributing behavioral transitions were then compared for morphs within similar contexts: (1) with only females, (2) in male dyads, and (3) in mixed sex groups. Frequencies for these transitions were first calculated for each male. These sampled frequencies were then compared using Wilcoxon signed-rank tests for the female group context and using Kruskal-Wallis ANOVA followed by Wilcoxon-signed rank pairwise tests for the male dyads and mixed sex contexts. Significant differences between social contexts were determined using Bonferroni-adjusted P-values. All statistical analyses and data visualizations were completed in R (v.4.2.3; R Core Team, 2021) and Adobe *Illustrator* (2023).

3.4 Results

3.4.1 Description of Male Eastern Mosquitofish Behaviors

To standardize behavioral analysis of male mosquitofish morphs, I define an ethogram of 20 social behaviors (Fig. 1; Table 1). Except for copulation, male- and female-directed behaviors are indistinguishable. Four new behaviors are described, extending the repertoire of previous *Gambusia* ethograms (Itzkowitz, 1971; Peden, 1972; Kraft *et al.*, 2018): “face-to-face” display, circling, wiggle, and “cut-off”.

The first three principal components (PCs) explain 52% of the variance in the data: 27%, 17%, and 8% (Fig. S4). Based on comparison of loadings and contributions (Fig. S5-6, Table S1), the behaviors that uniquely correlated with each PC could be determined. Assertive behaviors (chases, frontal displays, and lateral displays) correlate with PC1. Submissive behaviors (bitten, move away, and escape) as well as the assertive behavior “circling” correlate with PC2. Copulation, bites, and lunges are oppositely correlated from gonopodial extensions, “cut-off”, and skipping for PC3. Overall, most assertive and submissive behaviors group together in opposite directions on PC1 and PC2 (Fig. 2).

3.4.2 Female-Directed Behaviors Across Contexts

In a group with only females, mottled males are more assertive towards females than silver males are, but morphs show no differences in submission or dominance (Fig. 3; Tables S2-7). This difference in assertive attention is not explained by changes in types of behavior or transitions used (Fig. 4-5; Tables S8-15, S19-21); therefore, mottled males exhibit inherently higher overall assertive attention towards females than silver males do.

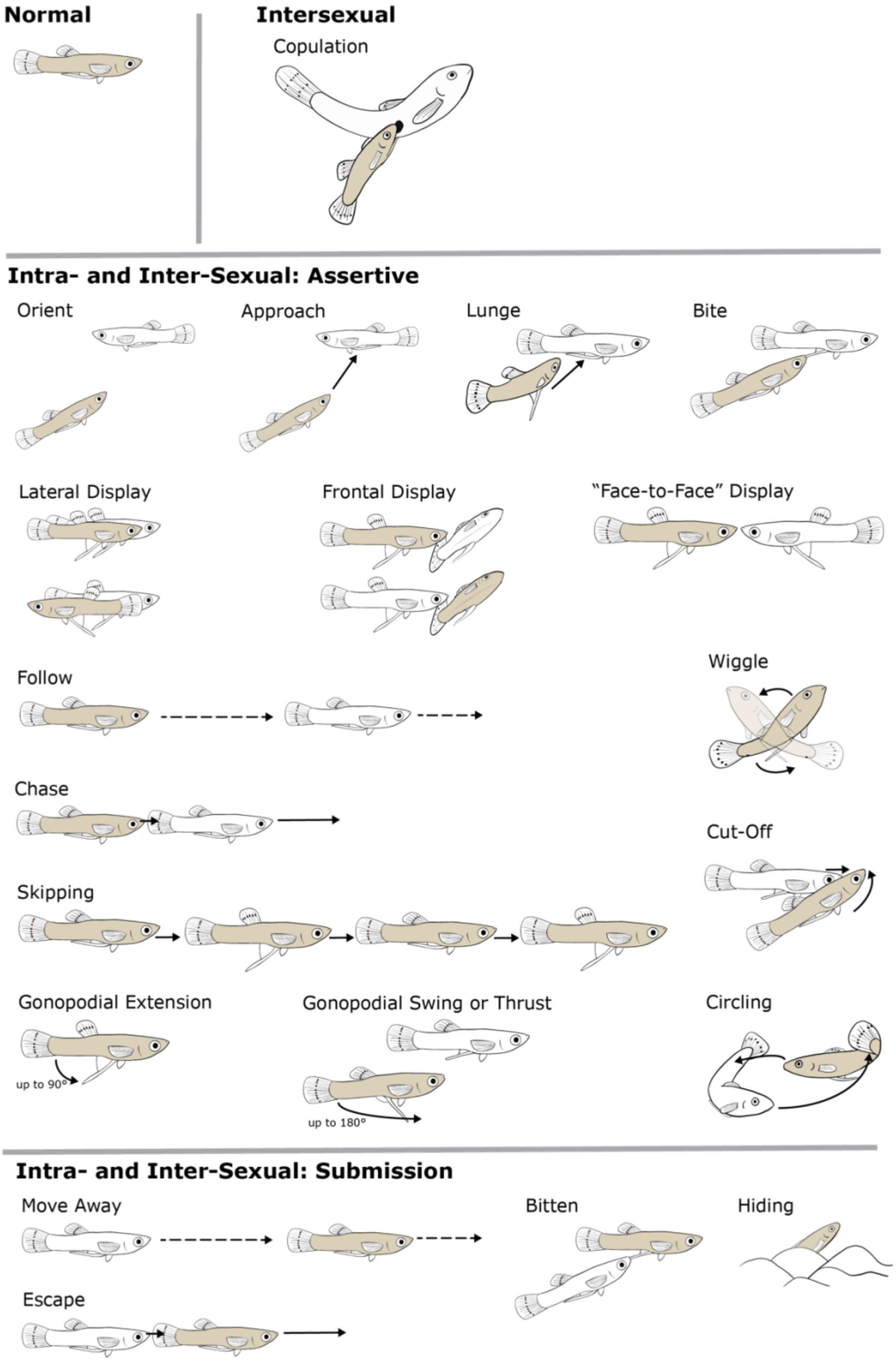


Figure 1. Pictorial description of male Eastern Mosquitofish (*Gambusia holbrooki*) social behaviors. The focal male is colorized, while the social partner is white-filled. Arrows indicate direction of movement, for which dashed and solid lines indicate slow and rapid movement, respectively.

Table 1. Description of the social behaviors exhibited by both mottled and silver Eastern Mosquitofish (*Gambusia holbrooki*) males. Only one behavior (copulation) was identified as unique to intersexual behavior. Assertive and submissive behaviors are exhibited towards both male (intrasexual) and female (intersexual) social partners. State events are behaviors that occur over a duration of time and can therefore be measured using both frequency and duration. Point events are behaviors that occur so quickly that only their frequency can be recorded.

Behavior Name	**Description**	**Sex of Social Partner**	**Event Type**
Normal	Focal male does not interact socially with another fish. He may be motionless (staying in place) or swimming in the behavioral arena.	none	state
Intersexual			
Copulation	Focal male swims alongside a female. In a rapid motion, he swings his gonopodium to the side or to the front. He inserts it into the female's gonopore. Insertion can be hard to capture on video, but the arched back of the female indicates that copulation occurred and can be used to distinguish this behavior from simple gonopodial swings.	female	state
Intra- and Inter-sexual: Assertive Attention			
Orient	Focal male points his head in the direction of the other fish. Longer durations of orient include staying stationary while maintaining this alignment with another fish. Orient does not include facing a conspecific while the focal male is in hiding.	both	state
Approach	Focal male moves towards a stationary fish. The movement may be slow or rapid.	both	state
Lunge	Focal male swims just behind and below the other fish and rapidly moves forward to make contact with the other fish. This movement is directed at another fish's ventral side, specifically female anal fins and male gonopodia.	both	state
Bite	Focal male bites the other fish (mouth touches the other fish's body). Bites are usually directed to the dorsal side of the body, caudal fins, female anal fins, and male gonopodia. This action often co-occurs with a state behavior.	both	point

Lateral Display	Focal male and another fish pass by each other, swim with each other, or remain stationary in a close parallel orientation. The interacting fish can face either the same or opposite directions. In extreme displays, a male will also lower his gonopodium.	both	state
Frontal Display	Focal male is stationary in a perpendicular or anterolateral orientation to the other fish. The focal male's body axis is oriented at about 90° to the other fish's body axis. The focal male usually spreads his dorsal and caudal fins. In extreme displays, a male will also lower his gonopodium.	both	state
"Face-to-Face" Display	After approaching the other fish, the focal male remains stationary in front of the other fish. This display occurs in a "face-to-face" position where the focal male's and other fish's heads are oriented towards each other and their snouts are close together. In extreme displays, a male will also lower his gonopodium.	both	state
Follow	Focal male swims behind the other fish. The focal male maintains a distance of at least one body length between himself and the other fish. The speed of movement is slow or medium.	both	state
Chase	Focal male swims directly behind the other fish. Usually, the focal male appears to be almost touching the other fish. The speed of movement is rapid.	both	state
Skipping	Focal male exhibits an extremely rapid succession of both swimming and gonopodial extension. He swims a short distance quickly (bursts) and then extends his gonopodium for a moment (a few milliseconds) before repeating this sequence multiple times.	both	state
Wiggle	Focal male creates a sinusoidal body movement by rapidly moving its pectoral and caudal fins while staying in place.	both	state
Cut-Off	Focal male swims rapidly in a short burst to position himself in front of another fish, thereby blocking the other fish and forcing it to change direction or stop swimming.	both	state
Gonopodial Extension	Focal male extends his gonopodium down (distance of extension is variable) and away from the body while also raising his dorsal fin.	both	state

Gonopodial Swing or Thrust	Focal male swings his extended gonopodium to the side of his body. The gonopodium is visibly perpendicular to the focal male's body. This action often co-occurs with a state behavior.	both	point
Circling	Both fish chase each other's caudal fins in a circular pattern (looped movement) in rapid motion.	both	state
Intra- and Inter-sexual: Submission			
Move Away	Focal male swims away from the opponent male after an aggressive action. This behavior is intermediate between normal swimming and escape. Often characterized by an initial rapid movement (a few milliseconds) that is not maintained.	both	state
Escape	Focal male swims away rapidly from the other fish while being followed or chased.	both	state
Bitten	The other fish bites the focal male (the other fish's mouth touches the focal male's body), usually on his caudal fin or gonopodium.	both	point
Hiding	Focal male stays in place in the substrate with little to no fin movement. Fish sometimes explore the substrate as part of normal behavior, but exploration is characterized by quick positional changes among the substrates.	both	state

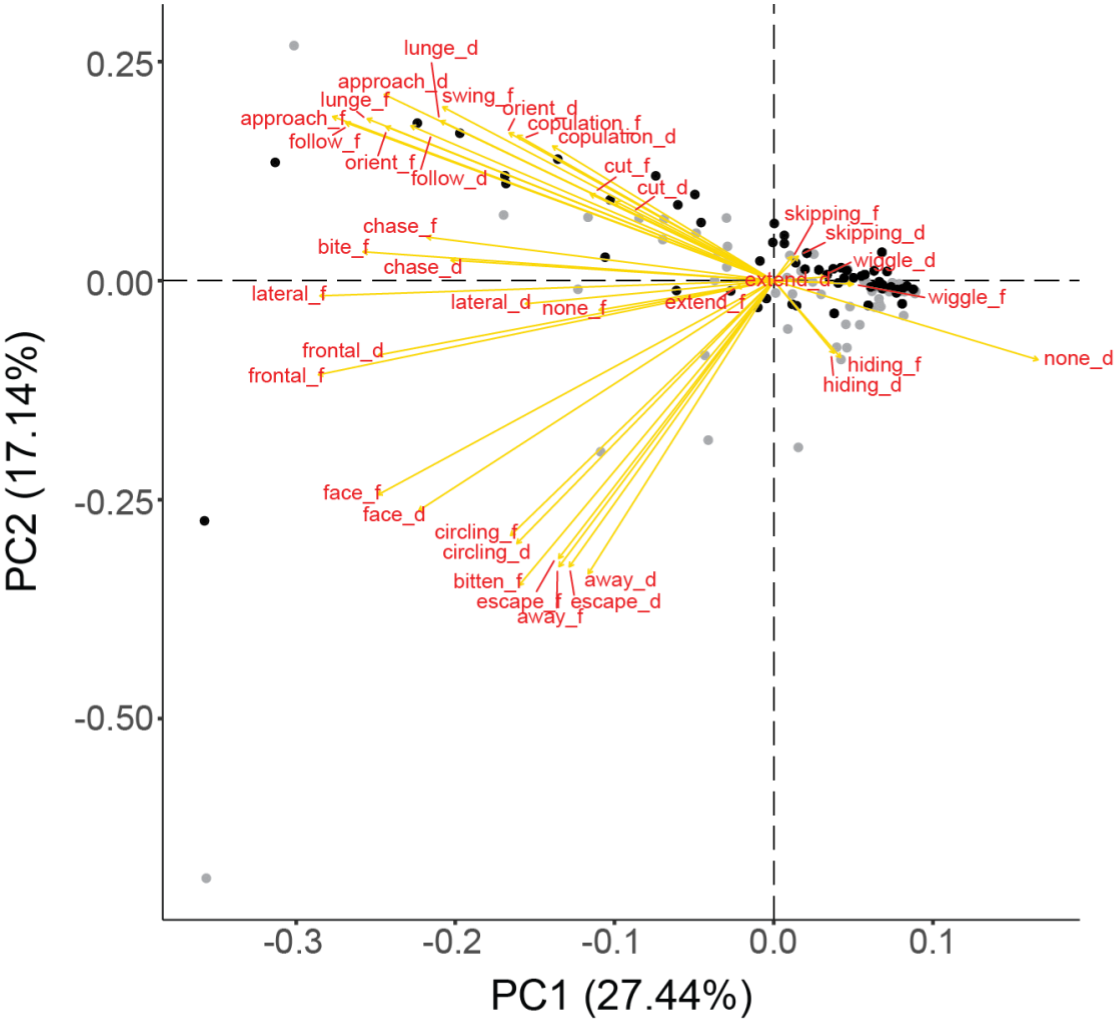


Figure 2. Bivariate plot showing the loading of all behavioral variables onto PC1 and PC2. Yellow arrows represent vectorized loadings. Dashed black lines show the 0 point for both PC1 and PC2. Focal fish behavior across contexts is represented on the plot for both mottled (black) and silver (gray) males. Most assertive behaviors grouped together, except for a select few ("face-to-face" display and circling) that grouped with submissive behaviors.

In mix-sex groups, however, the male morphs do not differ in assertive attention; mottled males lower their high assertive attention towards females to match the levels of silver males (Fig. 3A; Tables S2-3). Both morphs exhibit context-dependent changes in behavioral repertoire. Compared to when they are alone, silver males have a higher PC2 and mottled males have a lower PC3 towards females when a same-color male was present (Fig. 4; Tables S10-13). Specifically, silver males exhibit less circling and fewer of some submissive behaviors (bitten, move away, escape) towards females only when other silver males are also present; meanwhile, mottled males display fewer gonopodial extensions, cut-offs, and skipping, but more wiggles and copulations towards females when other mottled males are also present. Taken together, silver males decrease submissive behaviors while mottled males decrease some assertive behaviors towards females in the presence of a same-color male. Both morphs exhibit differences in behavioral transitions based on male phenotype (Fig. 7; Tables S19-21); they both use the same subset of five transitions with silver males but not with mottled males. Three transitions are from female-directed assertive behaviors to male-directed submissive behaviors: *chase (female)* → *move away (male)*, *follow (female)* → *hiding (male)*, and *frontal display (female)* → *hiding (male)*, while two transitions are from male-directed assertive behaviors to female-directed submissive behaviors: *hiding (male)* → *chase (female)*, and *move away (male)* → *chase (female)*. Morph-specific behavioral responses in female-directed behavior depends on both the social context and the phenotype of interacting males.

Within mix-sex groups, mosquitofish males show morph-specific, context-dependent changes in which behaviors they exhibit towards females. Silver males decrease PC2 in the presence of mottled males compared to silver males, while mottled males showed no differences based on male observer color (Fig. 4B; Tables S10-11). This decrease means that silver males use more circling and more of some submissive behaviors (bitten, move away, escape) towards females in the presence of a mottled male. Although silver males are using more of a subset of submissive behaviors, this change in behavior does not translate to a strong difference in overall submission or dominance towards females between morphs in mix-sex groups (Fig. 3; Tables S4-7).

3.4.3 Male-Directed Behaviors Across Contexts

No morph is inherently more aggressive or submissive towards other males because their behavior towards silver males, the most common male social partner in the wild, does not differ in dyads or in mix-sex groups (Fig. 3; Tables S2-13; S16-21). However, in dyads, silver males exhibit context-dependent changes in male-directed behavior while mottled males do not.

Silver males show less assertive attention, more submission, and less dominance to mottled males than silver males (Fig. 3; Tables S2-7). Silver males use less of the assertive transition *follow* → *orient* with mottled males than with silver males (Fig. 6; Tables S16-S18), which corresponds with the decreased assertive attention. Silver males also use the assertive transition *follow* → *orient* in same-color dyads more than mottled males do (Fig. 6; Tables S16-18), indicating a morph-specific behavioral response. Silver males have a lower PC3 (more wiggling; less gonopodial extension, cut-off, and skipping) towards mottled males than mottled males do towards other mottled males (Fig. 4C; Tables S12-S13), indicating that silver males depress certain assertive behaviors with mottled male opponents that mottled males do not. The increase in submission was best explained by the decrease in PC2 (Fig. 4B; Tables S10-11), which correlates with an increase in silver males being bitten by as well as moving away and escaping from mottled males. Silver males also use more of the submissive transition *move away* → *extend* with mottled males than with silver males, and more than mottled males use it with silver males (Fig. 6; Tables S19-21). On the other hand, mottled males do not change behavior based on opponent color (Fig. 3-4; Tables S19-21). Therefore, silver males but not mottled males exhibit a context-dependent change in male-directed behavior, which results in mottled males dominating silver males.

All males decrease male-directed assertive attention in the presence of females (Fig. 3A; Tables S2-3), likely due to interest in females diverting attention from opponent males. However, only mottled males strongly alter the behaviors that they use with males in the presence of females, and these behavioral changes are dependent on the interacting male's phenotype. In the presence of females, mottled males increase PC1 (fewer chases, frontal displays, and lateral displays) towards silver males, and lower PC3 (more wiggles; less gonopodial extension, cut-off, and skipping) towards mottled males (Fig. 4; Tables S8-9, S12-13). Mottled males exhibit a morph-specific behavioral response in the depression of their male-directed assertive behavior that depends on social context and the phenotype of interacting males.

Although differences in assertive attention and submission are no longer present in mix-sex groups, silver males still have a lower dominance index when interacting with mottled males (Fig. 3C; Tables S6-7), indicating that this measure of male-directed behavior is robust to changes in social context. In mix-sex groups, the differences in use of specific behaviors by silver males towards mottled males seen in dyads is no longer detected (Fig. 4; Tables S8-13). However, both morphs use a set of behavioral transitions with silver males that they do not with mottled males (Fig. 7; Tables S19-21); one transition is between submissive behaviors (*hiding* → *move away*), while two transitions are between assertive behaviors (*gonopodial extension* → *lateral display*, *gonopodial* *swing* → *lateral display*). Both morphs exhibit a change in behavioral sequence that depends on the phenotype of the interacting male in mix-sex groups.

3.4.4 Comparison of Female- and Male-Directed Behavior in Mix-Sex Contexts

Both morphs show more assertive attention towards females than towards males in mix-sex groups (Fig. 3; Tables S2-3). Generally, males have a higher PC1 towards males than females (Fig. 4A; Tables S8-9), which indicated that males direct more chases, frontal displays, and lateral displays towards females than males; however, this behavioral response is depressed in silver males in the presence of mottled males (Fig. 4A; Tables S8-9). Males have a lower PC2 towards males than females (Fig. 4B; Tables S10-11), meaning that males circle more with males than females and are bitten more by and moved away and escaped more from males than females. This response is also depressed in silver males in the presence of mottled males (Fig. 4B; Tables S10-11). Both morphs change their behavior based on sex of the fish in mix-sex groups, but only silver males show a depression in these changes when paired with mottled males, indicating a morph-specific response that depends on the phenotype of interacting males.

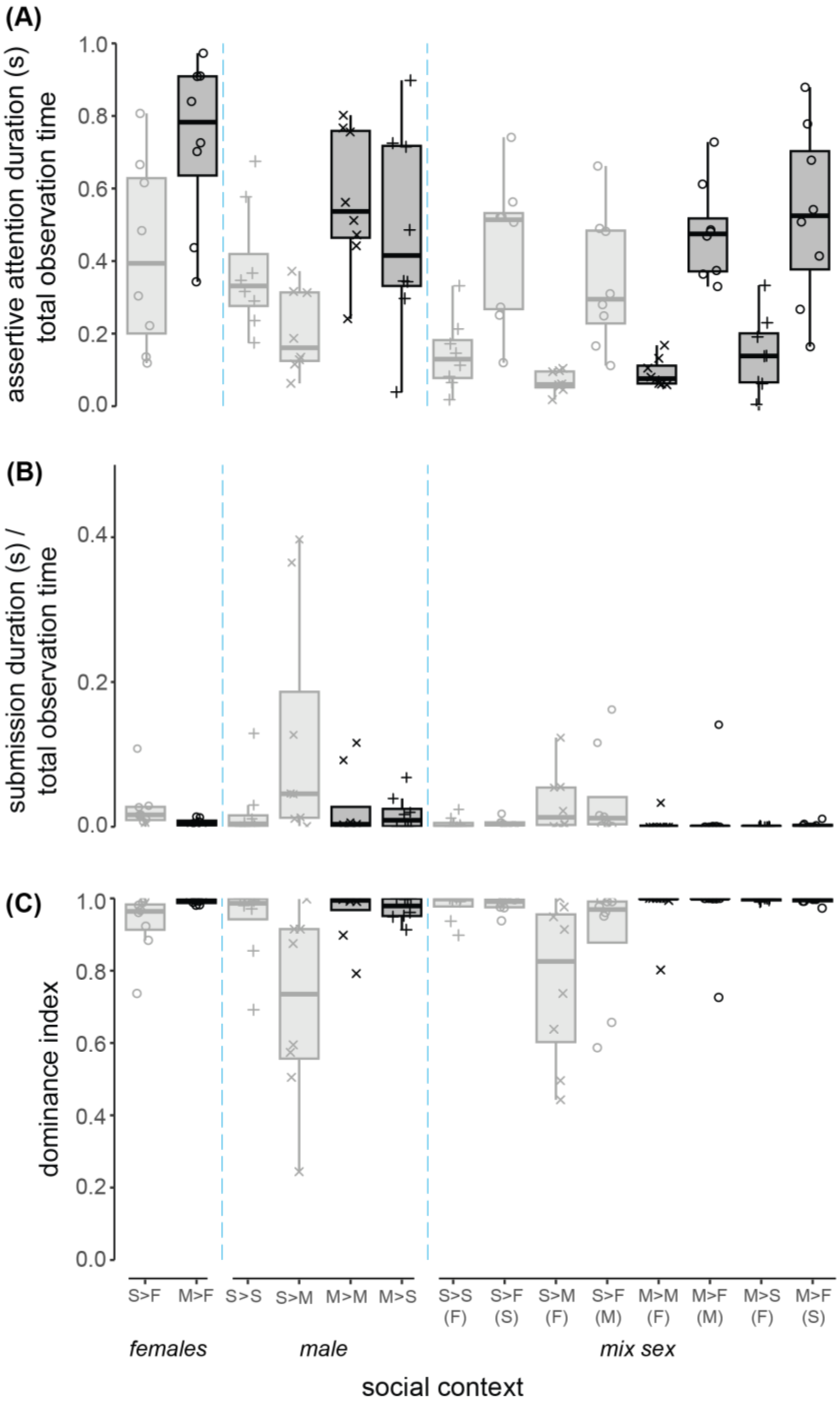


Figure 3. Assertive attention, submission, and dominance index of male mosquitofish morphs across social contexts. Social context labels are focal male > stimulus fish (observer fish). Color indicates focal male morph (gray for silver males, black for mottled males). Shape indicates stimulus fish: silver (S, plus), mottled (M, cross), and female (F, circle). **(A)** Mottled males show more assertive attention to females than silver males but lower it to the same level as silver males in mix-sex groups. Silver males depress their assertive attention with mottled males in dyads. All males direct more assertive attention towards females than males in mix-sex groups. **(B)** Only silver males with mottled males in dyadic interactions display more submission. **(C)** Silver males with mottled males in dyads and mix-sex groups express lower dominance.

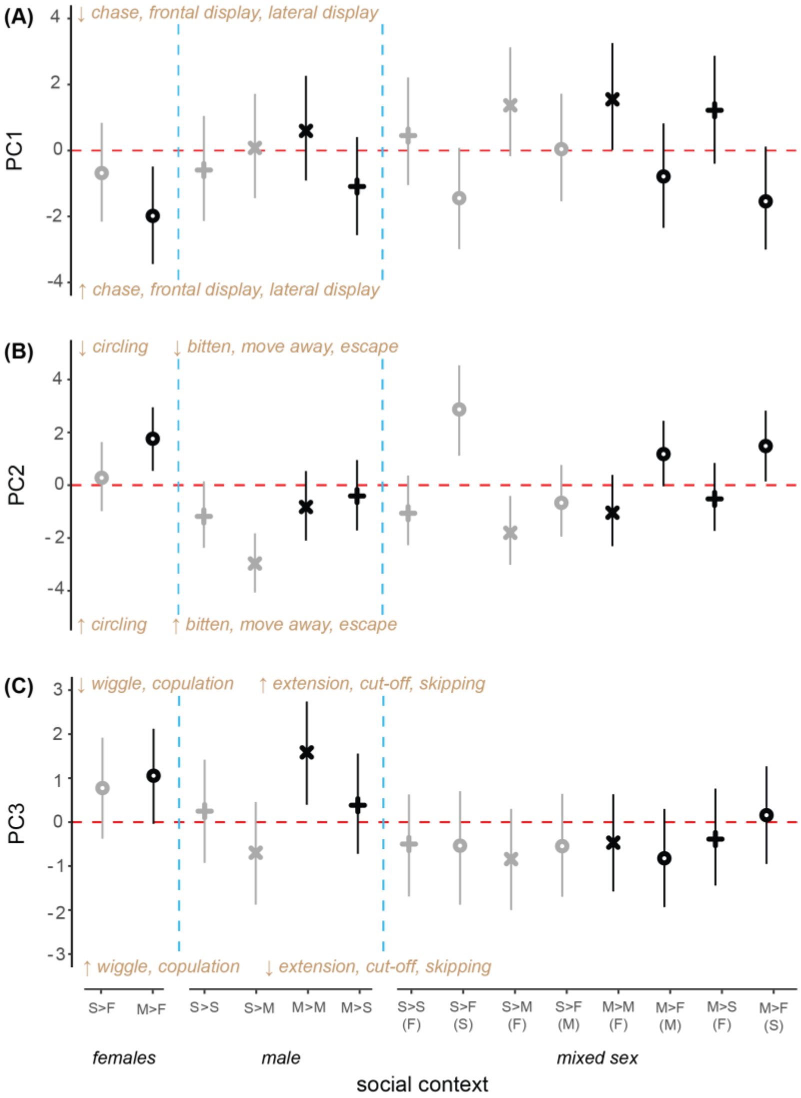


Figure 4. Specific behaviors expressed by male mosquitofish morphs across social contexts. Marginal median estimates for the first three PCs are shown. Lines represent 0.95 credible intervals based on highest posterior probability densities. The red dashed line at 0 is used to demarcate between higher and lower expression of behaviors. Social context labels are focal male > stimulus fish (observer fish). Color indicates focal male morph (silver = gray, mottled = black). Shape indicates stimulus fish: silver (S, plus), mottled (M, cross), and female (F, circle). **(A)** A lower PC1 means that males use more chases, frontal displays, and lateral displays. Generally, all males have a lower PC1 (more assertive) towards females than males in mix-sex groups, but silver males in the presence of mottled males do not. Mottled males have a higher PC1 (less assertive) towards silver males in mix-sex groups compared to male dyads. **(B)** A lower PC2 means that males circled their social partners more, but also were more submissive (bitten, moved away, and escaped). Silver males with mottled males have the lowest PC2 (more submissive) towards males in dyads and both sexes in mix-sex groups. Generally, all males have a higher PC2 (less submissive) towards females than males in mix-sex groups, but silver males in the presence of mottled males do not. Silver males have a higher PC2 (less submissive) towards females in the presence of silver males than when alone with females. **(C)** A higher PC3 means that males used more extensions, cut-off, and skipping, but less wiggles and copulations. Without female and mottled male observers, mottled males have a higher PC3 towards females (less copulation, more assertive) and towards mottled males (more assertive). In dyads, mottled males have a higher PC3 (more assertive) than silver males towards mottled males.

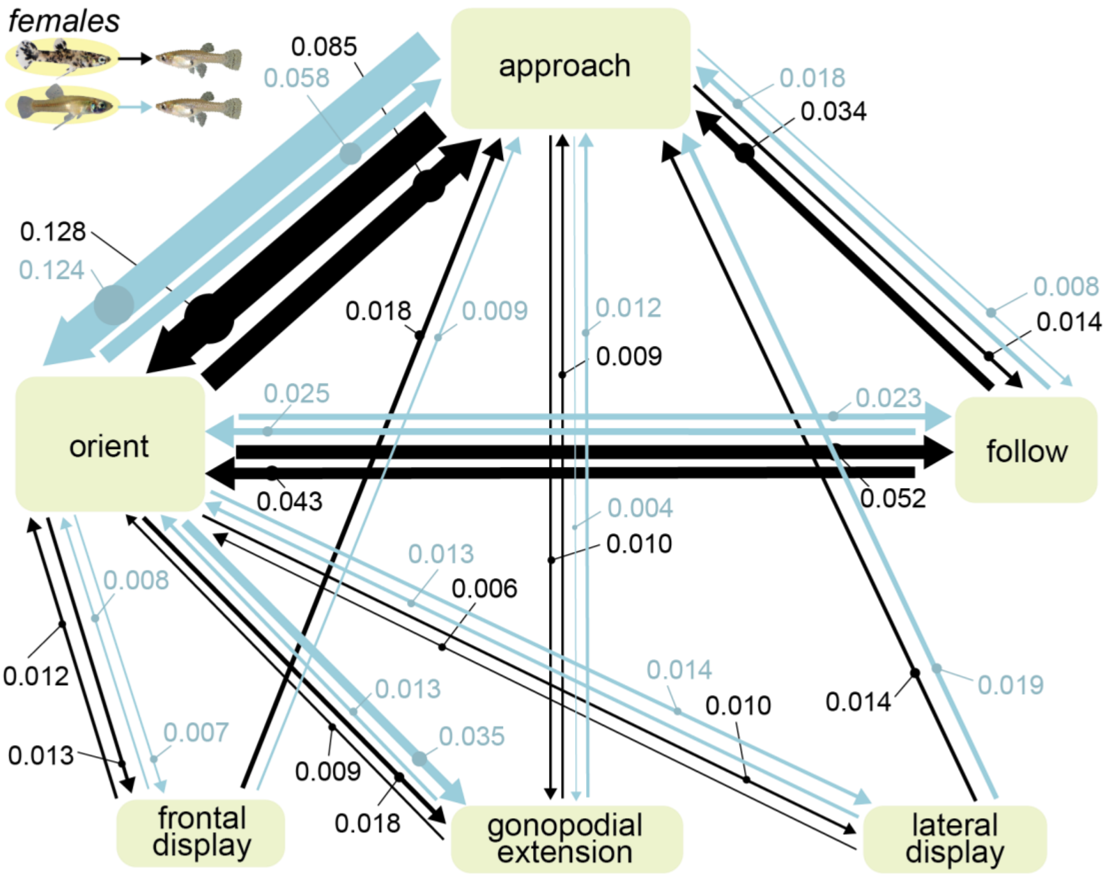


Figure 5. Transitions that significantly contribute to the behavioral sequence of male mosquitofish color morphs towards females. Frequencies are calculated based on all possible transitions. Arrow weight is proportional to frequency. Mottled males are black, while silver males are blue. Morphs do not significantly differ in behavioral sequence towards females.

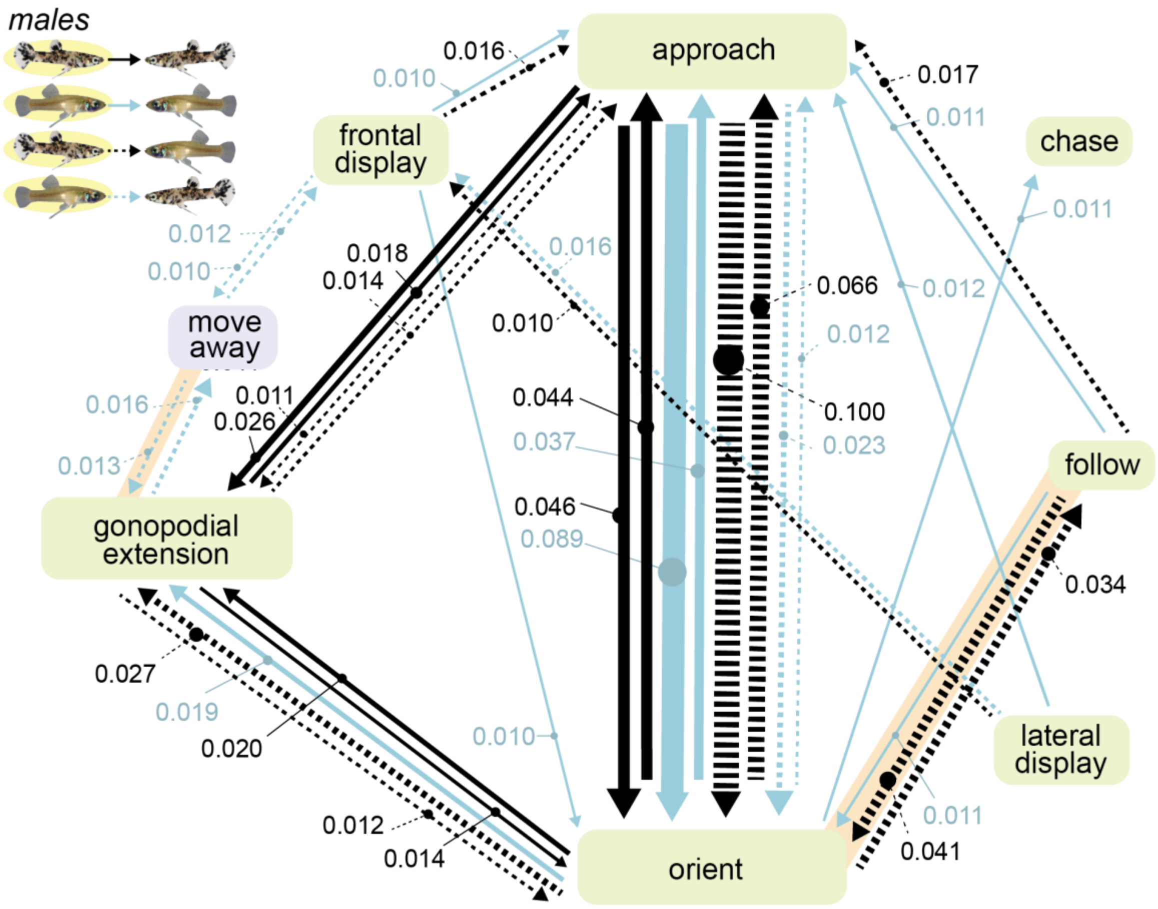


Figure 6. Transitions that significantly contribute to the behavioral sequence of male mosquitofish color morphs in male dyadic interactions. Frequencies are calculated based on all possible transitions. For simplicity, only significantly contributing frequencies >0.01 are shown here. Mottled males are black, while silver males are blue. Solid lines are towards a same-color opponent and dashed lines are towards a different-color opponent. Orange boxes indicate significant differences in behavioral sequence. Silver males use the transition *follow* → *orient* with silver males more than mottled males. Silver males use more *move away* → *gonopodial extension* with mottled males than with silver males.

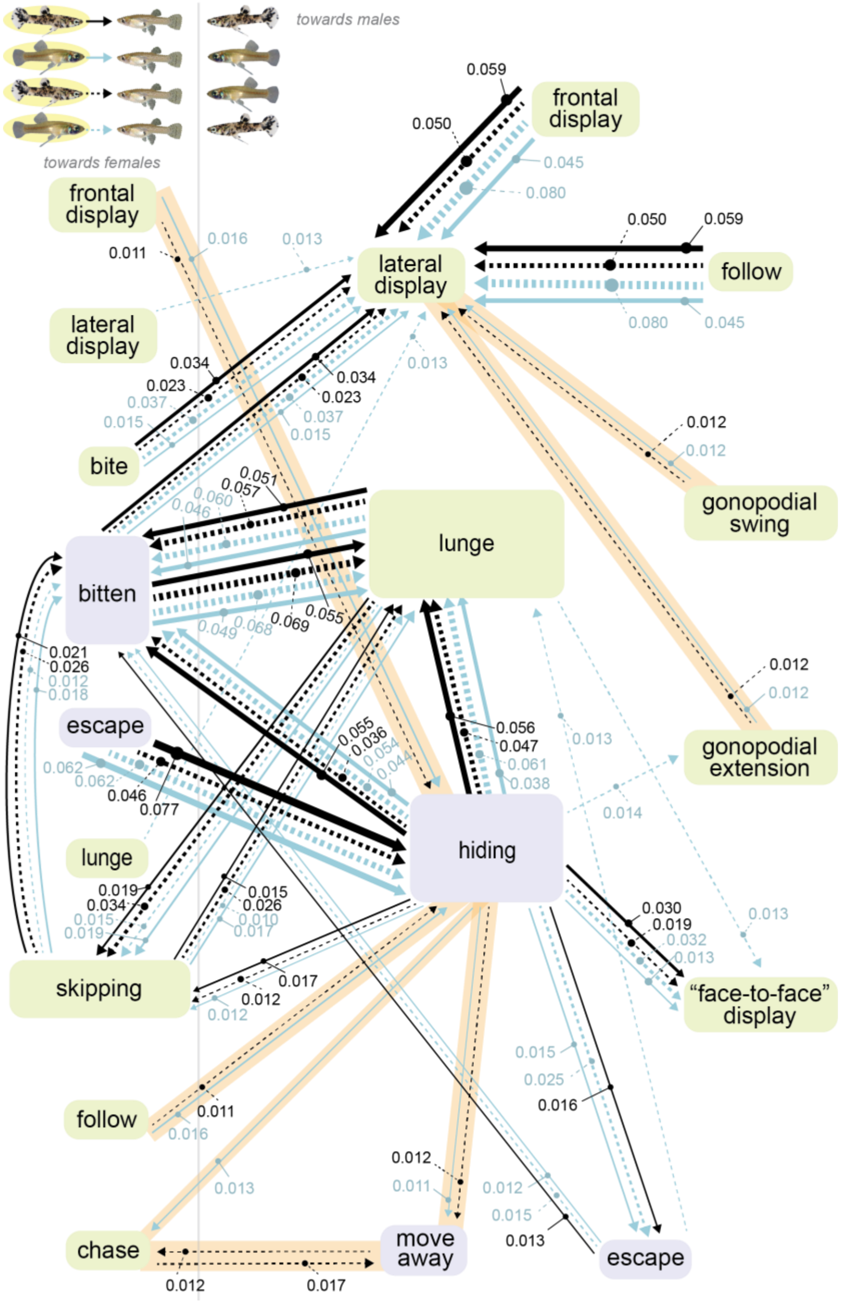


Figure 7. Transitions that significantly contribute to the behavioral sequence of male mosquitofish color morphs in mix-sex groups. Frequencies are calculated based on all possible transitions. For simplicity, only significantly contributing frequencies >0.01 are shown here. Arrow weight is proportional to frequency. Mottled males are black, while silver males are blue. Solid lines are contexts with same-color males and dashed lines are contexts with different-color males. Behaviors left of the gray line are towards females and right of it are towards males. Orange boxes indicate significant differences in behavioral sequence. Both morphs used a subset of transitions in contexts with silver males that they did not in contexts with mottled males. Three transitions are from female-directed assertive behaviors to male-directed submissive behaviors and two transitions are from male-directed assertive behaviors to female-directed submissive behaviors. For behavioral transitions only between male-directed behaviors, two transitions were between assertive behaviors while one transition was between submissive behaviors.

3.5 Discussion

3.5.1 Standardization of Male Eastern Mosquitofish Behavioral Experiments

I present the first comprehensive description of male social behaviors for *G. holbrooki*. Like previous *Gambusia* species ethograms (Itzkowitz, 1971; Peden, 1972), I did not find distinguishable differences in female- or male-directed social behaviors, but I did identify and describe four new behaviors. The PCA determined that most assertive behaviors (bite, swing, frontal display, lateral display, orient, approach, lunge, follow, chase) and submissive behaviors (bitten, move away, escape) group in opposing directions onto the first two PCs. This reduced subset of 12 behaviors could be used in situations where it is difficult to record and measure behavior, such as live observations in the field. Duration for a subset of active assertive behaviors correlated with total duration of assertive attention, so a reduced behavior set may be faster and easier to score while still being accurate; however, using a reduced behavior set could make it harder to capture differences in how specific behaviors are used by male mosquitofish, if that matters.

3.5.2 Female-Directed Behaviors Across Contexts

Mottled males are more assertive towards females than silver males when alone but not in mix-sex groups. Previous studies that used mix-sex groups also found either higher female-directed assertive attention from mottled males (Horth, 2003; Culumber *et al.*, 2020) or no difference between morphs (Martin, 1977; Karplus and Algom, 1996). Mottled and silver males exhibit different female-directed responses in individual behavioral actions depending on the social context; specifically, responses depended on whether another male was present and on that male’s color morph. Therefore, the conflicting results are likely due to the behavioral measurements and social groups used in each study. Within mix-sex groups, silver males use more fleeing-type behaviors with females in the presence of mottled males than of silver males. Similarly, silver juvenile mosquitofish flee more from females in the presence of mottled males than silver males (Kraft *et al.*, 2018), suggesting that this morph-specific, context-dependent behavioral response is repeatable.

When alone with females, mottled males are inherently more assertive than silver males. This melanin-based color pattern may be covarying with social behavior through a shared genetic, physiological, and/or developmental pathway (rev: Ducrest *et al.*, 2008; San-Jose and Roulin, 2018; Zerulla and Stoddard, 2021). Darker males usually display higher sexual activity in animals (rev: Ducrest *et al.*, 2008; San-Jose and Roulin, 2018), including for melanin-based color polymorphisms. Darker Stone's sheep (*Ovis dalli stonei*) show more tending behavior, meaning that they follow and defend females more (Loehr *et al*., 2008). Similarly, melanic male morphs of the Asian lady beetle (*Harmonia axyridis*) and the fly *Drosophila jambulina* have longer copulation periods than typical male morphs (Parkash *et al.*, 2009; Su *et al.*, 2013). For Penduline Tits (*Remiz pendulinus*), males with larger black masks pair more quickly than males with smaller masks (Kingma *et al*., 2008). Therefore, differences in sexual activity between male mosquitofish morphs is part of a larger pattern of phenotypic correlation between melanin-based color patterns and mating behavior across taxa.

Higher sexual activity by mottled morphs could lead to higher mating success than silver males (Martin, 1977). However, this higher sexual activity towards females is not maintained in mix-sex groups. The mottled morph may inconsistently experience the social environment in which they have a higher reproductive success compared to silver males. Future studies should examine how often mottled males experience each social environment in the field and how these contexts specifically affect morph reproductive success (e.g., number of matings, number of broods sired, and brood size).

3.5.3 Male-Directed Behaviors Across Contexts

No morph is inherently more aggressive or submissive, but mottled males dominate silver males. This dominance is driven by silver males depressing their assertive behaviors and increasing their submissive behaviors with mottled males relative to silver males; mottled males do not change their behavior based on the interacting male's phenotype. Horth (2003) similarly found that morphs do not differ in aggression towards silver males in mix-sex contexts, but mottled males direct more aggression towards silver males than they receive in return. This result is also consistent with findings for juvenile mosquitofish morphs in mix-sex groups (Kraft *et al*., 2018). Mottled juveniles do not differ in aggression whether they are with silver or mottled adults; however, silver males show more submission with mottled adults than with silver adults. The social dominance of mottled males is thus consistent and repeatable across ages, studies, and social contexts.

Across taxa, melanin-based color polymorphisms have been found to correlate with higher aggression, which is not the case for mosquitofish morphs, but also with social dominance (rev: Ducrest *et al*., 2008; San-Jose and Roulin, 2018). Darker Stone's sheep (*Ovis dalli stonei*) are socially more dominant (Loehr *et al*., 2008). Male Bluefin Killifish (*Lucania goodei*) with larger melanin outlines on their anal fins are also socially dominant (Johnson and Fuller, 2015). Black morphs of the Brazilian lizard *Tropidurus semitaeniatu*s exhibit both more aggressive displays and social dominance over yellow morphs (Bruinjé *et al.*, 2019). In birds, larger melanin ornaments are often associated with higher aggression and social dominance (rev: Jawor and Breitwisch, 2003; first determined for Harris's sparrows *Zonotrichia querula* by Rohwer, 1975). In insects, paper wasps (*Polistes dominulus*) with more black spots in their facial pattern are more likely to be dominant (Tibbets and Dale, 2004). Even without an inherently higher level of aggression, the mottled mosquitofish morph is still part of a larger trend in melanin-based color patterns covarying with social dominance.

Mottled males dominate silver males in mix-sex groups, but this dominance is not associated with significantly lower assertive attention and higher submission by silver males as it is in male dyads. In *Gambusia* species, dominant males usually exhibit continuous aggression while subordinate males do not exhibit strong submission, more commonly depressing their aggression in the presence of a dominant male (Caldwell and Caldwell, 1962; Itzkowitz, 1971). A change in behavioral strategy can be detected by my dominance index, which is calculated using relative differences between assertive attention and submission. Although I did not detect differences in assertive attention between morphs in mix-sex groups, dominance index was lower for silver males with mottled males, so I was still able to detect the change in behavioral strategy. Within dyads and mix-sex contexts, silver males change the types and transitions of behaviors used more than mottled males do. Mottled males appear to use the dominant strategy of not changing behavior regardless of the interacting male's morph, while silver males with mottled males use the subordinate strategy of changing their behavior. Silver juvenile mosquitofish also change their behavior more over time than mottled juvenile mosquitofish (Kraft *et al.*, 2018). Therefore, mottled males may not be as behaviorally flexible as silver males. If mottled males cannot modulate their behavior as well as silver males, they may maintain the dominant strategy regardless of the interacting individual's phenotype or the social context. However, mottled males reduce their use of male-directed behaviors when a female is present, and specific changes in behavior use also depend on the other male's morph. Mottled males can modulate their behavior, but they do so in a way that does not affect their dominance over silver males. Although color morph predicts social dominance for male mosquitofish, morph in this study was a combination of color pattern, social behaviors, and any unmeasured correlated traits; future studies should aim to separate color pattern from behavioral responses to test their individual effects on social dominance in male mosquitofish.

The mottled morph is stably maintained at a low frequency within some mosquitofish populations (Horth, 2004; Horth and Panayotova, 2012; Zerulla and Stoddard, 2021). Uncommon morphs are expected to go locally extinct through genetic drift (Wright, 1939; rev: Brockmann, 2001; Brisson, 2018). However, a rare-morph selective advantage may maintain the polymorphism in a population (Wright, 1939; e.g., Sinervo and Lively, 1996; Dijkstra *et al.*, 2010; rev: Brockmann, 2001; Brisson, 2018). Social dominance may provide a selective advantage to individuals if it is associated with better access to mates and other resources (West-Eberhard, 1979), although it is unclear whether this advantage exists for male mosquitofish morphs. Under this paradigm, the rare socially dominant morph would do well when it first enters a population, either through mutation or migration; however, as the morph's frequency increases, the rare-morph advantage is lost or exceeded by other disadvantages, ultimately being regulated by negative frequency-dependent selection to a frequency at which both morphs are evolutionarily stable (rev: Brockmann, 2001; Brisson, 2018). Other factors, such as predation, may exhibit stronger selective pressures on morph equilibrium frequency than social dominance (Horth, 2004; Horth and Panayotova, 2012; rev: Zerulla and Stoddard, 2021). It is also possible that social dominance of mottled males over silver males does not confer a rare-morph selective advantage in mosquitofish populations. Further studies are required to understand whether and how social dominance affects the relative fitness of mottled and silver male mosquitofish at varying morph frequencies.

3.5.4 Conclusions

Social behavior of male mosquitofish morphs depends on a combination of inherent genetic differences, the sex of the interacting fish, the morph of an interacting male (i.e., color pattern and its correlated social behaviors), and the social context. When interacting with only females, mottled males are more assertive than silver males; however, in mix-sex groups, mottled males lower their high assertive attention towards females to match silver males. In both dyads and mix-sex groups, mottled males dominate silver males. Neither morph appears to be inherently more aggressive, as the dominance of mottled males derives from silver males acting less aggressive and more submissive around mottled males. Both morphs are behaviorally flexible, and they alter their use of specific behaviors and transitions based on the interacting individual’s phenotype and the social context. In conclusion, variation in social environment affects the expression of social behavior differently for each male mosquitofish morph.

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3.7 Appendix

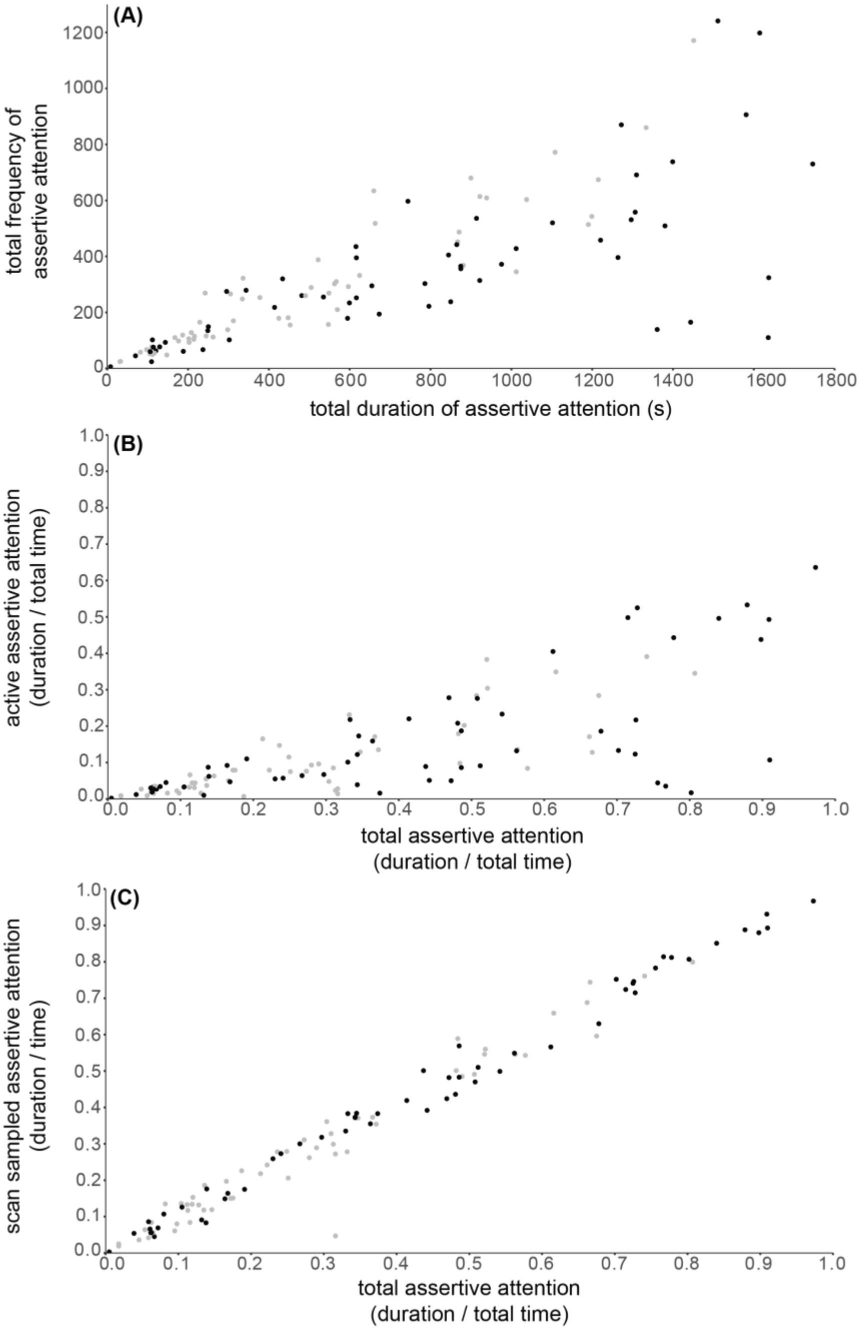

Figure S1. Evaluation of different methods of measuring social behavior for male mosquitofish. Using a Spearman's Rank Correlation Test, behavioral measurement methods from previous studies were all validated as accurate by significantly correlating with overall assertive attention ($P<0.001$). **(A)** Total frequency and total duration (s) of assertive behaviors were strongly correlated ($\rho = 0.86$). Total proportion of time spent exhibiting all active assertive behaviors was correlated with **(B)** the proportion of time spent exhibiting active assertive behaviors (approach, follow, chase, circling; $\rho = 0.77$), and **(C)** the proportion of time spent exhibiting all active assertive behaviors from scan-sampled time points ($\rho = 0.98$). Correlations did not appear to differ by morph (mottled = black; silver = gray; n = 56 per morph).

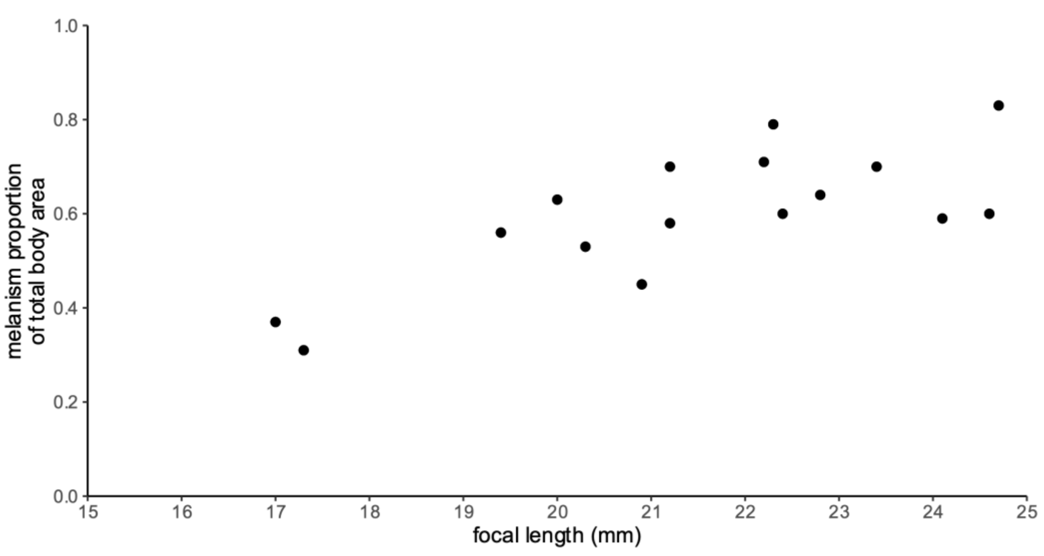

Figure S2. Size and melanism are correlated for the mottled male mosquitofish sampled in this study. Standard length (mm) and proportion of melanin on the total body area of mottled male mosquitofish (n=16) were significantly correlated (Spearman's Rank Correlation Test: ρ = 0.70, P<0.001). These two measures were thus confounding and their separate effects on mottled male social behavior could not be investigated in this study.

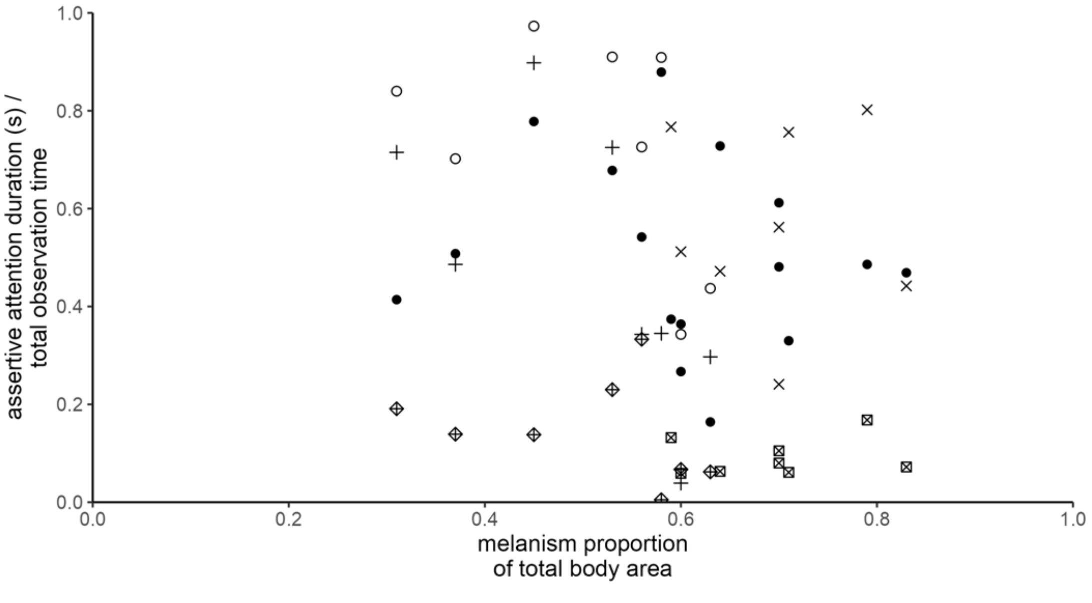

Figure S3. Relationship between melanism and total assertive attention expressed by mottled male mosquitofish. No obvious trends are apparent between the proportion of melanin on the total body area of mottled males and the proportion of total time spent exhibiting assertive attention within any social context. Shape indicates the social context and the interacting social partner's phenotype: females only (open circle), females in mix-sex groups (closed circle), silver male (plus), silver male in mix-sex group (diamond with a plus), mottled male (cross), and mottled male in mix-sex group (square with a cross).

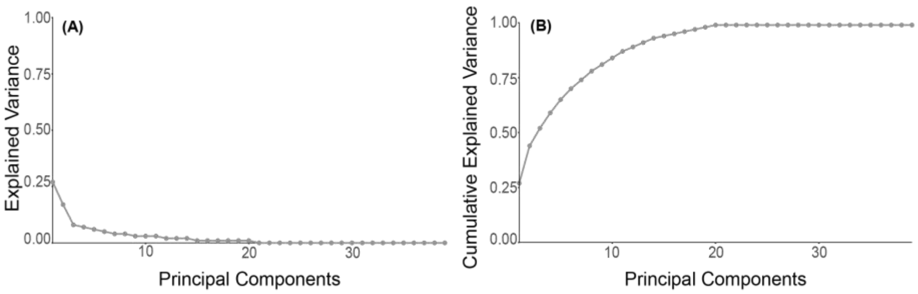

Figure S4. Scree plots for the explained variance and cumulative explained variance of the 39 PCs. The first three PCs explained approximately 54% of the variance in the data.

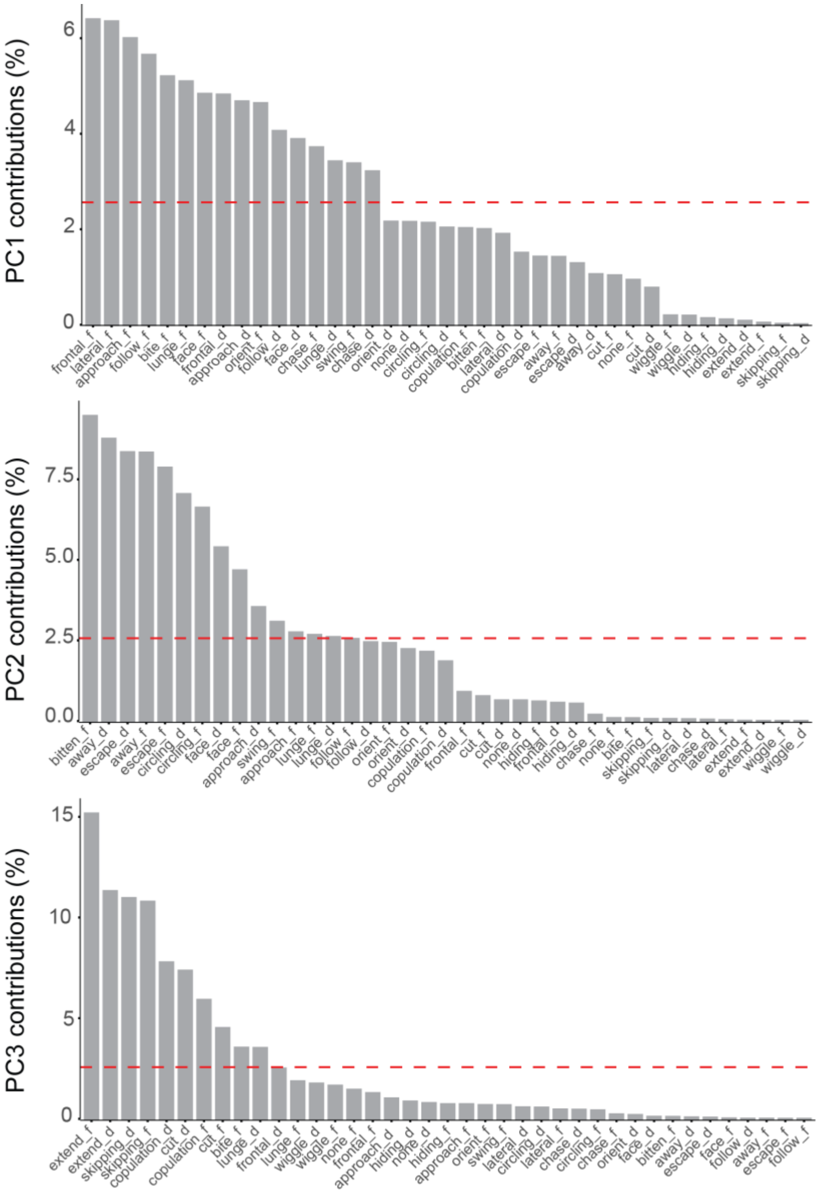

Figure S5. Ordered contributions (%) of each behavioral variable to the first three principal components (PCs). For each variable, f and d denote frequency and duration, respectively. Significant contribution was set to 2.5%, as indicated by the red dashed line. Strongly contributing behaviors were considered as bring primarily responsible for changes in that PC.

Table S1. Loadings of each behavioral variable on to the first three PCs. Frequency (f) and duration (d) of behavior are indicated under type. Contributions greater than 2.5% from Fig. S5 are highlighted in bold. Overall, most assertive and submissive behaviors group together in opposite directions on PC1 and PC2.

behavior	type	PC1	PC2	PC3
approach	f	**-0.24**	**0.17**	0.09
	d	**-0.22**	**0.19**	0.10
bite	f	**-0.23**	0.03	**-0.19**
chase	f	**-0.19**	0.04	-0.05
	d	**-0.18**	0.02	-0.07
follow	f	**-0.24**	**0.16**	-0.00
	d	**-0.20**	0.16	-0.01
gonopodial extension	f	-0.02	-0.01	**0.39**
	d	0.03	0.00	**0.34**
“cut-off”	f	-0.10	0.09	**0.21**
	d	-0.09	0.08	**0.27**
lunge	f	**-0.23**	**0.16**	-0.14
	d	**-0.18**	**0.16**	**-0.19**
orient	f	**-0.22**	0.16	0.08
	d	-0.15	0.15	0.04
skipping	f	0.01	0.02	**0.33**
	d	0.01	0.02	**0.33**
gonopodial swing or thrust	f	**-0.18**	**0.18**	-0.08
circling	f	-0.15	**-0.26**	-0.06
	d	-0.14	**-0.27**	-0.07
wiggle	f	0.04	-0.00	-0.13
	d	0.04	-0.00	-0.13
“face-to-face” display	f	**-0.22**	**-0.22**	0.01
	d	**-0.20**	**-0.23**	0.03
frontal display	f	**-0.25**	-0.09	0.11
	d	**-0.22**	-0.08	0.16
lateral display	f	**-0.25**	-0.02	0.07
	d	-0.14	-0.02	0.08
escape	f	-0.12	**-0.28**	0.00
	d	-0.11	**-0.29**	-0.02
hiding	f	0.04	-0.08	-0.09
	d	0.03	-0.07	-0.09
move away	f	-0.12	**-0.29**	-0.01
	d	-0.10	**-0.30**	-0.03
bitten	f	-0.14	**-0.31**	-0.03
copulation	f	-0.14	0.15	**-0.24**
	d	-0.12	0.14	**-0.28**
non-social	f	-0.10	-0.03	0.12
	d	0.15	-0.08	-0.09

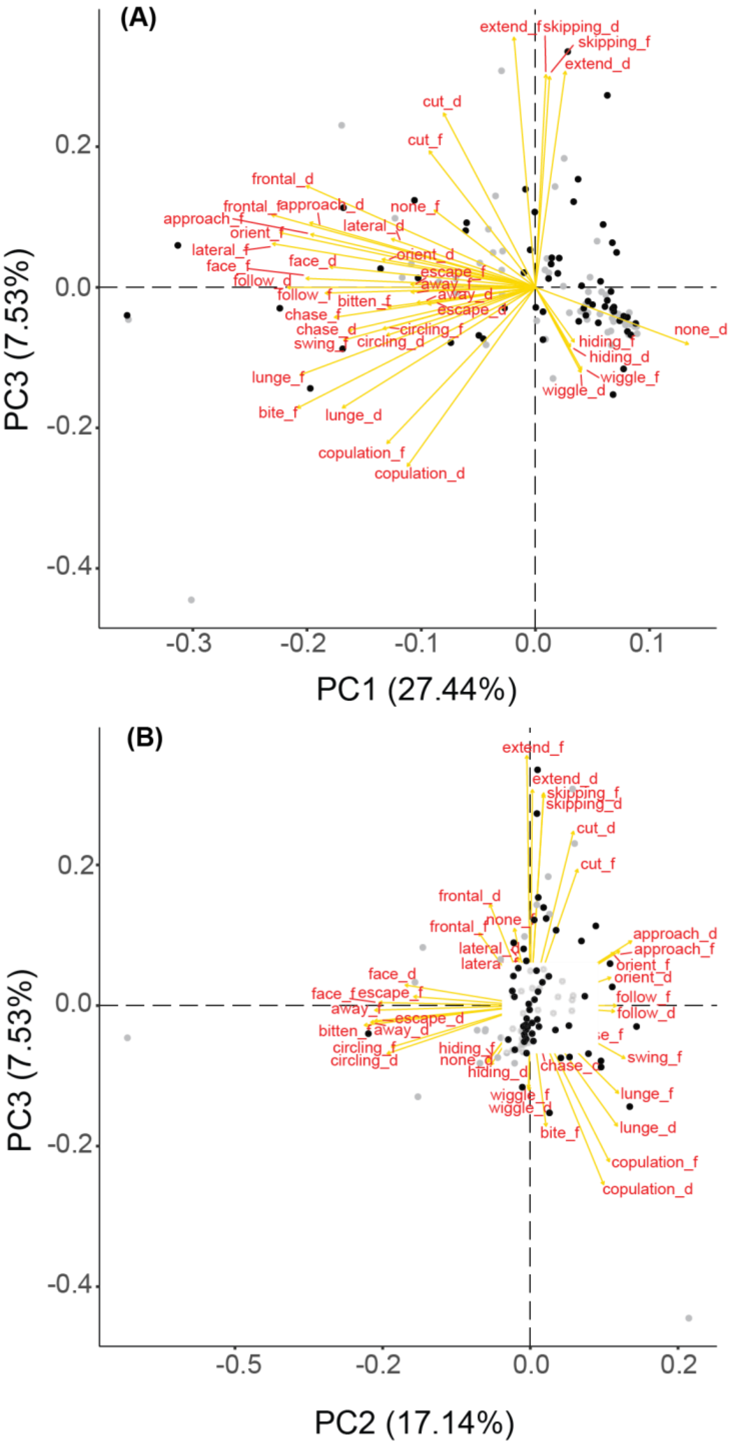

Figure S6. Remaining bivariate plots for the first three PCs: **(A)** PC1 and PC3, and **(B)** PC2 and PC3. Yellow arrows represent vectorized loadings. Dashed black lines show the 0 point for both PCs. Focal fish behavior across contexts is represented on the plot for both mottled (black) and silver (gray) males.

Table S2. Bayesian mixed effects model for assertive attention using a beta regression. Estimates, errors, and 95% credible intervals (CI) are presented for the random effect of subject and all fixed effects. Levels for each fixed effect are defined in parentheses: female (f), silver (s), mottled (m), and none (x).

Formula: assertive attention ~ focal * stimulus * observer + (1 | subject)
Φ ~ (1 | subject)

	estimate	**error**	**CI**
Bayes R^2	0.74	0.03	/
Random			
sd (Intercept)	0.64	0.14	0.42 – 0.95
sd (Φ Intercept)	0.92	0.31	0.39 – 1.60
Fixed			
Intercept	-0.76	0.44	-1.65 – 0.10
Φ Intercept	2.67	0.28	2.15 – 3.24
focal (s)	-0.44	0.45	-1.32 – 0.45
stimulus (m)	-1.24	0.47	-2.16 – -0.32
stimulus (s)	-1.03	0.44	-1.89 – -0.19
observer (m)	0.71	0.47	-0.22 – 1.64
observer (s)	0.62	0.44	-0.23 – 1.48
observer (x)	1.66	0.42	0.84 – 2.48
focal (s) * stimulus (m)	-0.05	0.49	-1.00 – 0.91
focal (s) * stimulus (s)	0.34	0.45	-0.53 – 1.23
focal (s) * observer (m)	-0.11	0.47	-1.04 – 0.81
focal (s) * observer (s)	0.20	0.45	-0.69 – 1.08
focal (s) * observer (x)	-0.77	0.42	-1.60 – 0.06
stimulus (m) * observer (m)	0.00	1.00	-1.94 – 1.94
stimulus (s) * observer (m)	-0.01	1.00	-1.98 – 1.96
stimulus (m) * observer (s)	0.01	1.01	-1.96 – 1.98
stimulus (s) * observer (s)	0.00	1.00	-1.94 – 1.96
stimulus (m) * observer (x)	0.63	0.47	-0.29 – 1.54
stimulus (s) * observer (x)	0.08	0.46	-0.81 – 0.98
focal (s) * stimulus (m) * observer (m)	0.00	1.00	-1.97 – 1.99
focal (s) * stimulus (s) * observer (m)	0.00	1.00	-1.97 – 1.97
focal (s) * stimulus (m) * observer (s)	-0.01	1.00	-1.95 – 1.95
focal (s) * stimulus (s) * observer (s)	0.00	1.00	-1.97 – 1.96
focal (s) * stimulus (m) * observer (x)	-0.39	0.51	-1.41 – 0.62
focal (s) * stimulus (s) * observer (x)	0.47	0.50	-0.52 – 1.44

Table S3. Contrasts for assertive attention of male mosquitofish morphs across social contexts. Contrasts are based on pairwise comparisons of the median posterior probability estimates and 0.95 credible interval of highest posterior probability density (HPD) with multivariate *t*-distribution adjustment. Contexts are defined as focal male > stimulus fish (observer). Levels are defined as female (f), silver (s), and mottled (m). Important contrasts (i.e., HPD does not cross 0) are in bold. Mottled males show more assertive attention to females than silver males but lower it to the same level as silver males in mix-sex groups. Silver males depress their assertive attention with mottled males in dyads. All males direct more assertive attention towards females than males in mix-sex groups.

social context	contrast	estimate	lower HPD	upper HPD
females				
	m > f - s > f	**0.29**	**0.11**	**0.45**
mix-sex: females				
	m > f (m) - s > f (m)	0.13	-0.06	0.31
	m > f (m) - m > f (s)	0.02	-0.17	0.21
	m > f (m) - s > f (s)	0.08	-0.12	0.27
	s > f (m) - m > f (s)	-0.11	-0.30	0.08
	s > f (m) - s > f (s)	-0.05	-0.16	0.04
	m > f (s) - s > f (s)	0.06	-0.13	0.25
female comparison				
	m > f (m) - m > f	**-0.22**	**-0.39**	**-0.05**
	s > f (m) - s > f	-0.07	-0.18	0.03
	m > f (s) - m > f	**-0.24**	**-0.35**	**-0.12**
	s > f (s) - s > f	-0.02	-0.12	0.09
male-male dyads				
	m > m - s > m	**0.36**	**0.18**	**0.53**
	m > m - m > s	0.08	-0.12	0.28
	m > m - s > s	0.18	-0.02	0.38
	s > m - m > s	**-0.28**	**-0.46**	**-0.11**
	s > m - s > s	**-0.19**	**-0.30**	**-0.07**
	m > s - s > s	0.10	-0.10	0.29
mix-sex: males				
	m > m (f) - s > m (f)	0.04	-0.03	0.13
	m > m (f) - m > s (f)	-0.02	-0.12	0.07
	m > m (f) - s > s (f)	-0.01	-0.11	0.09
	s > m (f) - m > s (f)	-0.07	-0.16	0.02
	s > m (f) - s > s (f)	-0.05	-0.12	0.00
	m > s (f) - s > s (f)	0.01	-0.09	0.11
male comparison				
	m > m (f) - m > m	**-0.45**	**-0.57**	**-0.32**
	s > m (f) - s > m	**-0.12**	**-0.21**	**-0.05**
	m > s (f) - m > s	**-0.34**	**-0.46**	**-0.21**
	s > s (f) - s > s	**-0.26**	**-0.37**	**-0.14**
mix-sex: between sexes				
	m > m (f) - m > f (m)	**-0.36**	**-0.49**	**-0.24**
	s > m (f) - s > f (m)	**-0.27**	**-0.38**	**-0.17**
	m > s (f) - m > f (s)	**-0.32**	**-0.44**	**-0.20**
	s > s (f) - s > f (s)	**-0.27**	**-0.38**	**-0.16**

Table S4. Bayesian mixed effects model for submission using a beta regression. Estimates, errors, and 95% credible intervals (CI) are presented for the random effect of subject and all fixed effects. Levels for each fixed effect are defined in parentheses: female (f), silver (s), mottled (m), and none (x).

Formula: submission ~ focal * stimulus * observer + (1 | subject)
Φ ~ (1 | subject)

	estimate	**error**	**CI**
Bayes R^2	0.34	0.16	/
Random			
sd (Intercept)	0.81	0.32	0.13 – 1.41
sd (Φ Intercept)	1.39	0.71	0.10 – 2.70
Fixed			
Intercept	-5.09	0.61	-6.23 – -3.82
Φ Intercept	4.15	0.65	2.92 – 5.36
focal (s)	0.83	0.57	-0.27 – 1.95
stimulus (m)	-0.01	0.52	-1.02 – 0.99
stimulus (s)	-0.26	0.47	-1.16 – 0.67
observer (m)	0.14	0.53	-0.89 – 1.16
observer (s)	-0.12	0.49	-1.08 – 0.84
observer (x)	0.56	0.46	-0.35 – 1.44
focal (s) * stimulus (m)	0.50	0.55	-0.58 – 1.56
focal (s) * stimulus (s)	0.02	0.52	-1.00 – 1.03
focal (s) * observer (m)	0.23	0.57	-0.90 – 1.35
focal (s) * observer (s)	-0.01	0.55	-1.10 – 1.05
focal (s) * observer (x)	0.07	0.48	-0.85 – 1.00
stimulus (m) * observer (m)	0.00	1.01	-1.98 – 1.98
stimulus (s) * observer (m)	0.00	1.00	-1.95 – 1.95
stimulus (m) * observer (s)	0.00	1.01	-1.98 – 1.97
stimulus (s) * observer (s)	0.00	0.99	-1.94 – 1.94
stimulus (m) * observer (x)	0.12	0.51	-0.89 – 1.13
stimulus (s) * observer (x)	0.19	0.51	-0.82 – 1.22
focal (s) * stimulus (m) * observer (m)	0.00	1.00	-1.97 – 1.97
focal (s) * stimulus (s) * observer (m)	0.01	1.00	-1.97 – 1.97
focal (s) * stimulus (m) * observer (s)	0.00	1.00	-1.96 – 1.98
focal (s) * stimulus (s) * observer (s)	0.00	1.00	-1.98 – 1.93
focal (s) * stimulus (m) * observer (x)	0.40	0.60	-0.79 – 1.58
focal (s) * stimulus (s) * observer (x)	-0.12	0.61	-1.60 – 0.78

Table S5. Contrasts for submission of male mosquitofish morphs across social contexts. Contrasts are based on pairwise comparisons of the median posterior probability estimates and 0.95 credible interval of highest posterior probability density (HPD) with multivariate *t*-distribution adjustment. Contexts are defined as focal male > stimulus fish (observer). Levels are defined as female (f), silver (s), and mottled (m). Important contrasts (i.e., HPD does not cross 0) are in bold. Only silver males with mottled males in dyadic interactions display more submission.

social context	**contrast**	**estimate**	**lower HPD**	**upper HPD**
females				
	m > f - s > f	-0.01	-0.04	0.01
mix-sex: females				
	m > f (m) - s > f (m)	-0.01	-0.04	0.00
	m > f (m) - m > f (s)	0.00	-0.01	0.01
	m > f (m) - s > f (s)	0.00	-0.02	0.01
	s > f (m) - m > f (s)	0.01	0.00	0.04
	s > f (m) - s > f (s)	0.01	-0.01	0.03
	m > f (s) - s > f (s)	-0.01	-0.02	0.01
female comparison				
	m > f (m) - m > f	0.00	-0.01	0.01
	s > f (m) - s > f	-0.01	-0.03	0.02
	m > f (s) - m > f	0.00	-0.01	0.00
	s > f (s) - s > f	-0.01	-0.04	0.00
male-male dyads				
	m > m - s > m	**-0.05**	**-0.12**	**-0.01**
	m > m - m > s	0.00	-0.01	0.02
	m > m - s > s	0.00	-0.03	0.02
	s > m - m > s	**0.06**	**0.01**	**0.12**
	s > m - s > s	**0.05**	**0.01**	**0.10**
	m > s - s > s	-0.01	-0.03	0.01
mix-sex: males				
	m > m (f) - s > m (f)	-0.02	-0.04	0.00
	m > m (f) - m > s (f)	0.00	-0.01	0.01
	m > m (f) - s > s (f)	0.00	-0.02	0.01
	s > m (f) - m > s (f)	0.02	0.00	0.04
	s > m (f) - s > s (f)	0.01	0.00	0.03
	m > s (f) - s > s (f)	-0.01	-0.02	0.00
male comparison				
	m > m (f) - m > m	-0.01	-0.02	0.00
	s > m (f) - s > m	-0.04	-0.10	0.00
	m > s (f) - m > s	0.00	-0.02	0.00
	s > s (f) - s > s	-0.01	-0.02	0.01
mix-sex: between sexes				
	m > m (f) - m > f (m)	0.00	-0.01	0.01
	s > m (f) - s > f (m)	0.00	-0.02	0.02
	m > s (f) - m > f (s)	0.00	-0.01	0.00
	s > s (f) - s > f (s)	0.00	-0.01	0.01

Table S6. Bayesian mixed effects model for dominance index using a beta regression. Estimates, errors, and 95% credible intervals (CI) are presented for the random effect of subject and all fixed effects. Levels for each fixed effect are defined in parentheses: female (f), silver (s), mottled (m), and none (x).

Formula: dominance index ~ focal * stimulus * observer + (1 | subject)
Φ ~ (1 | subject)

	estimate	**error**	**CI**
Bayes R^2	0.24	0.09	/
Random			
sd (Intercept)	0.24	0.17	0.01 – 0.65
sd (Φ Intercept)	0.30	0.27	0.01 – 1.04
Fixed			
Intercept	3.34	0.53	2.36 – 4.45
Φ Intercept	1.94	0.30	1.47 – 2.70
focal (s)	-0.70	0.49	-1.70 – 0.22
stimulus (m)	-0.08	0.49	-1.04 – 0.87
stimulus (s)	0.11	0.48	-0.83 – 1.04
observer (m)	-0.03	0.51	-1.02 – 0.98
observer (s)	0.01	0.50	-0.96 – 1.00
observer (x)	-0.21	0.45	-1.10 – 0.66
focal (s) * stimulus (m)	-0.86	0.53	-1.89 – 0.17
focal (s) * stimulus (s)	0.26	0.53	-0.77 – 1.29
focal (s) * observer (m)	-0.29	0.57	-1.39 – 0.83
focal (s) * observer (s)	0.22	0.56	-0.89 – 1.32
focal (s) * observer (x)	-0.02	0.49	-0.98 – 0.93
stimulus (m) * observer (m)	0.00	0.99	-1.94 – 1.94
stimulus (s) * observer (m)	0.00	0.99	-1.96 – 1.96
stimulus (m) * observer (s)	0.00	1.00	-1.96 – 1.97
stimulus (s) * observer (s)	0.00	1.01	-2.00 – 1.98
stimulus (m) * observer (x)	-0.04	0.53	-1.08 – 1.00
stimulus (s) * observer (x)	-0.20	0.54	-1.25 – 0.85
focal (s) * stimulus (m) * observer (m)	0.01	1.00	-1.95 – 1.97
focal (s) * stimulus (s) * observer (m)	0.00	0.99	-1.95 – 1.96
focal (s) * stimulus (m) * observer (s)	-0.01	0.99	-1.96 – 1.95
focal (s) * stimulus (s) * observer (s)	0.00	1.00	-1.95 – 1.95
focal (s) * stimulus (m) * observer (x)	-0.17	0.62	-1.38 – 1.04
focal (s) * stimulus (s) * observer (x)	0.15	0.63	-1.09 – 1.38

Table S7. Contrasts for dominance index of male mosquitofish morphs across social contexts. Contrasts are based on pairwise comparisons of the median posterior probability estimates and 0.95 credible interval of highest posterior probability density (HPD) with multivariate *t*-distribution adjustment. Contexts are defined as focal male > stimulus fish (observer). Levels are defined as female (f), silver (s), and mottled (m). Important contrasts (i.e., HPD does not cross 0) are in bold. Silver males with mottled males in dyads and mix-sex groups express lower dominance.

social context	**contrast**	**estimate**	**lower HPD**	**upper HPD**
females				
	m > f - s > f	0.04	-0.01	0.10
mix-sex: females				
	m > f (m) - s > f (m)	0.05	0.00	0.12
	m > f (m) - m > f (s)	0.00	-0.04	0.03
	m > f (m) - s > f (s)	0.02	-0.03	0.07
	s > f (m) - m > f (s)	-0.05	-0.12	0.00
	s > f (m) - s > f (s)	-0.04	-0.11	0.03
	m > f (s) - s > f (s)	0.02	-0.02	0.06
female comparison				
	m > f (m) - m > f	0.01	-0.03	0.05
	s > f (m) - s > f	-0.01	-0.08	0.07
	m > f (s) - m > f	0.01	-0.03	0.04
	s > f (s) - s > f	0.03	-0.03	0.09
male-male dyads				
	m > m - s > m	**0.17**	**0.07**	**0.29**
	m > m - m > s	0.00	-0.05	0.05
	m > m - s > s	0.01	-0.04	0.07
	s > m - m > s	**-0.18**	**-0.29**	**-0.06**
	s > m - s > s	**-0.16**	**-0.28**	**-0.06**
	m > s - s > s	0.02	-0.04	0.07
mix-sex: males				
	m > m (f) - s > m (f)	**0.12**	**0.04**	**0.21**
	m > m (f) - m > s (f)	-0.01	-0.04	0.03
	m > m (f) - s > s (f)	0.01	-0.03	0.05
	s > m (f) - m > s (f)	**-0.12**	**-0.22**	**-0.04**
	s > m (f) - s > s (f)	**-0.11**	**-0.20**	**-0.03**
	m > s (f) - s > s (f)	0.02	-0.02	0.06
male comparison				
	m > m (f) - m > m	0.01	-0.03	0.05
	s > m (f) - s > m	0.07	-0.06	0.20
	m > s (f) - m > s	0.01	-0.02	0.05
	s > s (f) - s > s	0.02	-0.03	0.07
mix-sex: between sexes				
	m > m (f) - m > f (m)	0.00	-0.04	0.03
	s > m (f) - s > f (m)	-0.06	-0.17	0.03
	m > s (f) - m > f (s)	0.00	-0.03	0.04
	s > s (f) - s > f (s)	0.01	-0.04	0.05

Table S8. Bayesian mixed effects model for PC1 using a skewed normal distribution. Estimates, errors, and 95% credible intervals (CI) are presented for the random effect of subject and all fixed effects. Levels for each fixed effect are defined in parentheses: female (f), silver (s), mottled (m), and none (x).

Formula: PC1 ~ focal * stimulus * observer + (1 | subject)

	estimate	**error**	**CI**
Bayes R^2	0.35	0.08	/
Random			
sd (Intercept)	1.40	0.33	0.82 – 2.12
Fixed			
Intercept	-0.31	1.42	-3.09 – 2.49
focal (s)	0.38	1.43	-2.43 – 3.21
stimulus (m)	1.90	1.50	-1.01 – 4.90
stimulus (s)	1.55	1.45	-1.30 – 4.43
observer (m)	-0.46	1.53	-3.45 – 2.58
observer (s)	-1.20	1.47	-4.02 – 1.72
observer (x)	-1.67	1.41	-4.42 – 1.14
focal (s) * stimulus (m)	-0.57	1.53	-3.57 – 2.42
focal (s) * stimulus (s)	-1.13	1.48	-4.04 – 1.78
focal (s) * observer (m)	0.46	1.58	-2.65 – 3.57
focal (s) * observer (s)	-0.30	1.52	-3.30 – 2.71
focal (s) * observer (x)	0.93	1.42	-1.84 – 3.73
stimulus (m) * observer (m)	0.00	3.50	-6.85 – 6.89
stimulus (s) * observer (m)	0.00	3.53	-6.90 – 6.94
stimulus (m) * observer (s)	-0.01	3.50	-6.89 – 6.85
stimulus (s) * observer (s)	-0.01	3.50	-6.83 – 6.90
stimulus (m) * observer (x)	0.70	1.54	-2.34 – 3.72
stimulus (s) * observer (x)	-0.65	1.51	-3.63 – 2.32
focal (s) * stimulus (m) * observer (m)	0.01	3.49	-6.81 – 6.86
focal (s) * stimulus (s) * observer (m)	0.00	3.49	-6.85 – 6.84
focal (s) * stimulus (m) * observer (s)	0.00	3.51	-6.88 – 6.82
focal (s) * stimulus (s) * observer (s)	0.00	3.52	-6.91 – 6.85
focal (s) * stimulus (m) * observer (x)	-1.27	1.68	-4.54 – 2.05
focal (s) * stimulus (s) * observer (x)	0.31	1.64	-2.93 – 3.51

Table S9. Contrasts for PC1 of male mosquitofish morphs across social contexts. Contrasts are based on pairwise comparisons of the median posterior probability estimates and 0.95 credible interval of highest posterior probability density (HPD) with multivariate *t*-distribution adjustment. Contexts are defined as focal male > stimulus fish (observer). Levels are defined as female (f), silver (s), and mottled (m). Important contrasts (i.e., HPD does not cross 0) are in bold. A lower PC1 means that males use more chases, frontal displays, and lateral displays. Generally, all males have a lower PC1 (more assertive) towards females than males in mix-sex groups, but silver males in the presence of mottled males do not. Mottled males have a higher PC1 (less assertive) towards silver males in mix-sex groups compared to male dyads.

social context	contrast	estimate	lower HPD	upper HPD
females				
	m > f - s > f	-1.31	-3.37	0.73
mix-sex: females				
	m > f (m) - s > f (m)	-0.83	-3.08	1.37
	m > f (m) - m > f (s)	0.74	-1.43	2.91
	m > f (m) - s > f (s)	0.66	-1.49	2.90
	s > f (m) - m > f (s)	1.58	-0.67	3.84
	s > f (m) - s > f (s)	1.49	-0.23	3.30
	m > f (s) - s > f (s)	-0.09	-2.23	2.09
female comparison				
	m > f (m) - m > f	1.19	-0.84	3.33
	s > f (m) - s > f	0.74	-0.95	2.51
	m > f (s) - m > f	0.46	-1.15	2.19
	s > f (s) - s > f	-0.76	-2.34	0.78
male-male dyads				
	m > m - s > m	0.53	-1.72	2.68
	m > m - m > s	1.70	-0.42	3.84
	m > m - s > s	1.20	-1.03	3.44
	s > m - m > s	1.17	-1.02	3.33
	s > m - s > s	0.67	-1.07	2.46
	m > s - s > s	-0.50	-2.64	1.64
mix-sex: males				
	m > m (f) - s > m (f)	0.18	-1.97	2.47
	m > m (f) - m > s (f)	0.35	-1.85	2.48
	m > m (f) - s > s (f)	1.10	-1.23	3.34
	s > m (f) - m > s (f)	0.15	-2.12	2.43
	s > m (f) - s > s (f)	0.92	-0.90	2.75
	m > s (f) - s > s (f)	0.74	-1.51	2.94
male comparison				
	m > m (f) - m > m	0.96	-0.74	2.73
	s > m (f) - s > m	1.30	-0.43	3.12
	m > s (f) - m > s	**2.33**	**0.57**	**4.07**
	s > s (f) - s > s	1.05	-0.63	2.93
mix-sex: between sexes				
	m > m (f) - m > f (m)	**2.35**	**0.52**	**4.26**
	s > m (f) - s > f (m)	1.32	-0.38	3.15
	m > s (f) - m > f (s)	**2.76**	**0.96**	**4.54**
	s > s (f) - s > f (s)	**1.90**	**0.21**	**3.72**

Table S10. Bayesian mixed effects model for PC2 using a skewed normal distribution. Estimates, errors, and 95% credible intervals (CI) are presented for the random effect of subject and all fixed effects. Levels for each fixed effect are defined in parentheses: female (f), silver (s), mottled (m), and none (x).

Formula: PC2 ~ focal * stimulus * observer + (1 | subject)

	estimate	**error**	**CI**
Bayes R^2	0.36	0.05	/
Random			
sd (Intercept)	0.45	0.27	0.02 – 1.03
Fixed			
Intercept	0.88	1.22	-1.51 – 3.27
focal (s)	-0.55	1.18	-2.85 – 1.75
stimulus (m)	-1.88	1.29	-4.38 – 0.64
stimulus (s)	-1.37	1.28	-3.86 – 1.13
observer (m)	0.32	1.31	-2.23 – 2.90
observer (s)	0.62	1.33	-1.98 – 3.23
observer (x)	0.88	1.24	-1.55 – 3.31
focal (s) * stimulus (m)	-0.20	1.31	-2.77 – 2.37
focal (s) * stimulus (s)	0.02	1.31	-2.57 – 2.60
focal (s) * observer (m)	-1.27	1.38	-3.94 – 1.46
focal (s) * observer (s)	1.91	1.46	-0.96 – 4.78
focal (s) * observer (x)	-0.91	1.26	-3.35 – 1.55
stimulus (m) * observer (m)	-0.01	3.02	-5.92 – 5.87
stimulus (s) * observer (m)	-0.01	3.00	-5.92 – 5.91
stimulus (m) * observer (s)	-0.01	3.00	-5.89 – 5.84
stimulus (s) * observer (s)	0.00	3.01	-5.88 – 5.58
stimulus (m) * observer (x)	-0.67	1.37	-3.36 – 2.02
stimulus (s) * observer (x)	-0.79	1.38	-3.48 – 1.93
focal (s) * stimulus (m) * observer (m)	-0.01	2.99	-5.85 – 5.84
focal (s) * stimulus (s) * observer (m)	0.00	2.99	-5.84 – 5.87
focal (s) * stimulus (m) * observer (s)	-0.01	2.99	-5.93 – 5.86
focal (s) * stimulus (s) * observer (s)	-0.01	3.02	-5.94 – 5.93
focal (s) * stimulus (m) * observer (x)	-0.50	1.51	-3.48 – 2.43
focal (s) * stimulus (s) * observer (x)	0.69	1.51	-2.26 – 3.62

Table S11. Contrasts for PC2 of male mosquitofish morphs across social contexts. Contrasts are based on pairwise comparisons of the median posterior probability estimates and 0.95 credible interval of highest posterior probability density (HPD) with multivariate *t*-distribution adjustment. Contexts are defined as focal male > stimulus fish (observer). Levels are defined as female (f), silver (s), and mottled (m). Important contrasts (i.e., HPD does not cross 0) are in bold. A lower PC2 means that males circled their social partners more, but also were more submissive (bitten, moved away, and escaped). Silver males with mottled males have the lowest PC2 (more submissive) towards males in dyads and both sexes in mix-sex groups. Generally, all males have a higher PC2 (less submissive) towards females than males in mix-sex groups, but silver males in the presence of mottled males do not. Silver males have a higher PC2 (less submissive) towards females in the presence of silver males than when alone with females.

social context	contrast	estimate	lower HPD	upper HPD
females				
	m > f - s > f	1.48	-0.30	3.14
mix-sex: females				
	m > f (m) - s > f (m)	1.84	0.00	3.56
	m > f (m) - m > f (s)	-0.30	-2.05	1.54
	m > f (m) - s > f (s)	-1.69	-3.82	0.49
	s > f (m) - m > f (s)	**-2.13**	**-4.07**	**-0.20**
	s > f (m) - s > f (s)	**-3.50**	**-5.75**	**-1.19**
	m > f (s) - s > f (s)	-1.38	-3.44	0.72
female comparison				
	m > f (m) - m > f	-0.58	-2.17	1.20
	s > f (m) - s > f	-0.94	-2.67	0.89
	m > f (s) - m > f	-0.27	-1.92	1.39
	s > f (s) - s > f	**2.56**	**0.43**	**4.70**
male-male dyads				
	m > m - s > m	**2.13**	**0.46**	**3.85**
	m > m - m > s	-0.41	-2.23	1.56
	m > m - s > s	0.35	-1.51	2.13
	s > m - m > s	**-2.56**	**-4.30**	**-0.85**
	s > m - s > s	**-1.77**	**-3.49**	**-0.26**
	m > s - s > s	0.77	-1.07	2.62
mix-sex: males				
	m > m (f) - s > m (f)	0.75	-1.03	2.51
	m > m (f) - m > s (f)	-0.52	-2.25	1.25
	m > m (f) - s > s (f)	0.02	-1.86	1.82
	s > m (f) - m > s (f)	-1.28	-3.05	0.59
	s > m (f) - s > s (f)	-0.73	-2.49	0.96
	m > s (f) - s > s (f)	0.54	-1.22	2.27
male comparison				
	m > m (f) - m > m	-0.22	-1.92	1.47
	s > m (f) - s > m	1.16	-0.44	2.79
	m > s (f) - m > s	-0.10	-1.89	1.67
	s > s (f) - s > s	0.11	-1.57	1.82
mix-sex: between sexes				
	m > m (f) - m > f (m)	**-2.22**	**-3.90**	**-0.48**
	s > m (f) - s > f (m)	-1.13	-2.90	0.62
	m > s (f) - m > f (s)	**-1.99**	**-3.79**	**-0.20**
	s > s (f) - s > f (s)	**-3.91**	**-6.16**	**-1.62**

Table S12. Bayesian mixed effects model for PC3 using a skewed normal distribution. Estimates, errors, and 95% credible intervals (CI) are presented for the random effect of subject and all fixed effects. Levels for each fixed effect are defined in parentheses: female (f), silver (s), mottled (m), and none (x).

Formula: PC3 ~ focal * stimulus * observer + (1 | subject)

	estimate	**error**	**CI**
Bayes R^2	0.42	0.06	/
Random			
sd (Intercept)	0.93	0.24	0.52 – 1.45
Fixed			
Intercept	1.11	0.86	-0.59 – 2.82
focal (s)	0.77	0.87	-0.95 – 2.49
stimulus (m)	-1.57	0.92	-3.36 – 0.25
stimulus (s)	-1.49	0.89	-3.23 – 0.24
observer (m)	-1.92	0.95	-3.77 – -0.05
observer (s)	-0.95	0.92	-2.75 – 0.85
observer (x)	-0.05	0.85	-1.74 – 1.62
focal (s) * stimulus (m)	-1.15	0.96	-3.00 – 0.75
focal (s) * stimulus (s)	-0.89	0.93	-2.70 – 0.92
focal (s) * observer (m)	-0.50	1.00	-2.45 – 1.47
focal (s) * observer (s)	-1.48	1.01	-3.48 – 0.50
focal (s) * observer (x)	-1.05	0.88	-2.79 – 0.66
stimulus (m) * observer (m)	-3.01	2.01	-6.96 – 0.92
stimulus (s) * observer (m)	-3.00	2.00	-6.91 – 0.93
stimulus (m) * observer (s)	-2.99	2.00	-6.93 – 0.96
stimulus (s) * observer (s)	-2.99	1.99	-6.92 – 0.89
stimulus (m) * observer (x)	2.10	0.99	0.15 – 4.03
stimulus (s) * observer (x)	0.82	0.96	-1.08 – 2.72
focal (s) * stimulus (m) * observer (m)	-3.01	1.99	-6.94 – 0.93
focal (s) * stimulus (s) * observer (m)	-3.00	2.00	-6.92 – 0.95
focal (s) * stimulus (m) * observer (s)	-2.99	1.99	-6.90 – 0.89
focal (s) * stimulus (s) * observer (s)	-3.00	1.99	-6.93 – 0.89
focal (s) * stimulus (m) * observer (x)	-0.86	1.10	-2.98 – 1.31
focal (s) * stimulus (s) * observer (x)	1.03	1.08	-1.08 – 3.16

Table S13. Contrasts for PC3 of male mosquitofish morphs across social contexts. Contrasts are based on pairwise comparisons of the median posterior probability estimates and 0.95 credible interval of highest posterior probability density (HPD) with multivariate *t*-distribution adjustment. Contexts are defined as focal male > stimulus fish (observer). Levels are defined as female (f), silver (s), and mottled (m). Important contrasts (i.e., HPD does not cross 0) are in bold. A higher PC3 means that males used more extensions, cut-off, and skipping, but less wiggles and copulations. Without female and mottled male observers, mottled males have a higher PC3 towards females (less copulation, more assertive) and towards mottled males (more assertive). In dyads, mottled males have a higher PC3 (more assertive) than silver males towards mottled males.

social context	contrast	estimate	lower HPD	upper HPD
females				
	m > f - s > f	0.28	-1.25	1.77
mix-sex: females				
	m > f (m) - s > f (m)	-0.27	-1.81	1.31
	m > f (m) - m > f (s)	-0.98	-2.53	0.52
	m > f (m) - s > f (s)	-0.29	-1.99	1.55
	s > f (m) - m > f (s)	-0.70	-2.31	0.92
	s > f (m) - s > f (s)	-0.01	-1.52	1.56
	m > f (s) - s > f (s)	0.70	-0.98	2.39
female comparison				
	m > f (m) - m > f	**-1.88**	**-3.35**	**-0.40**
	s > f (m) - s > f	-1.31	-2.68	0.00
	m > f (s) - m > f	-0.90	-2.20	0.35
	s > f (s) - s > f	-1.31	-2.79	0.10
male-male dyads				
	m > m - s > m	**2.28**	**0.68**	**4.00**
	m > m - m > s	1.20	-0.45	2.80
	m > m - s > s	1.34	-0.27	3.04
	s > m - m > s	-1.08	-2.71	0.60
	s > m - s > s	-0.95	-2.30	0.42
	m > s - s > s	0.14	-1.42	1.78
mix-sex: males				
	m > m (f) - s > m (f)	0.37	-1.12	1.94
	m > m (f) - m > s (f)	-0.08	-1.56	1.39
	m > m (f) - s > s (f)	0.03	-1.57	1.61
	s > m (f) - m > s (f)	-0.45	-2.08	1.09
	s > m (f) - s > s (f)	-0.34	-1.71	0.93
	m > s (f) - s > s (f)	0.12	-1.41	1.64
male comparison				
	m > m (f) - m > m	**-2.05**	**-3.40**	**-0.69**
	s > m (f) - s > m	-0.14	-1.48	1.12
	m > s (f) - m > s	-0.77	-2.06	0.49
	s > s (f) - s > s	-0.76	-2.04	0.63
mix-sex: between sexes				
	m > m (f) - m > f (m)	0.34	-0.91	1.64
	s > m (f) - s > f (m)	-0.30	-1.58	1.06
	m > s (f) - m > f (s)	-0.54	-1.83	0.74
	s > s (f) - s > f (s)	0.04	-1.44	1.59

Table S14. Transitions that contribute significantly to the social behavior sequence used by each male mosquitofish morph towards a group of females. Counts are pooled across trials. Frequencies are calculated based on all possible transitions. Z-scores are calculated based median absolute deviation for transitions with frequencies > 0. Only transitions with P-values less than the Bonferroni-adjusted alpha for at least one morph are included here.

focal	**transition**	**count**	**frequency**	**z**
silver	approach → follow	39	0.008	8.19
	approach → gonopodial extension	20	0.004	3.87
	approach → orient	612	0.124	138.53
	follow → approach	90	0.018	19.79
	follow → orient	124	0.025	27.52
	frontal display → approach	43	0.009	9.10
	frontal display → orient	42	0.008	8.87
	gonopodial extension → approach	57	0.012	12.28
	gonopodial extension → orient	66	0.013	14.33
	lateral display → approach	96	0.019	21.15
	lateral display → orient	66	0.013	14.33
	orient → approach	286	0.058	64.37
	orient → follow	115	0.023	25.48
	orient → frontal display	35	0.007	7.28
	orient → gonopodial extension	172	0.035	38.44
	orient → lateral display	70	0.014	15.24
mottled	approach → follow	87	0.014	9.33
	approach → gonopodial extension	61	0.010	6.37
	approach → orient	782	0.128	88.37
	follow → approach	206	0.034	22.86
	follow → orient	262	0.043	29.23
	frontal display → approach	108	0.018	11.71
	frontal display → orient	72	0.012	7.62
	gonopodial extension → approach	57	0.009	5.91
	gonopodial extension → orient	52	0.009	5.35
	lateral display → approach	85	0.014	9.10
	lateral display → orient	36	0.006	3.53
	orient → approach	518	0.085	58.35
	orient → follow	320	0.052	35.83
	orient → frontal display	79	0.013	8.42
	orient → gonopodial extension	109	0.018	11.83
	orient → lateral display	63	0.010	6.60

Table S15. Comparison of social behavior transitions used by male mosquitofish morphs towards a group of females. Behavioral transitions that contributed significantly to overall social behavioral sequence are compared between morphs using Wilcoxon signed-rank tests with a Bonferroni-adjusted alpha (0.003). Morphs do not differ significantly in use of behavioral transitions with groups of females.

transition	**V-stat**	**P-value**
approach → follow	15	0.06
approach → gonopodial extension	12	0.28
approach → orient	23	0.55
follow → approach	25	0.38
follow → orient	24	0.46
frontal display → approach	36	0.01
frontal display → orient	25	0.08
gonopodial extension → approach	18	0.55
gonopodial extension → orient	19	0.95
lateral display → approach	14	0.64
lateral display → orient	7	0.15
orient → approach	32	0.05
orient → follow	15	0.93
orient → frontal display	35	0.02
orient → gonopodial extension	17	0.95
orient → lateral display	17	0.95

Table S16. Transitions that contribute significantly to the social behavior sequence used by each male mosquitofish morph towards same- or different-color males in dyadic encounters. Counts are pooled across trials. Frequencies are calculated based on all possible transitions. Z-scores are calculated based median absolute deviation for transitions with frequencies > 0. Only transitions with P-values less than the Bonferroni-adjusted alpha for at least one dyad are included here.

focal	**stimulus**	**transition**	**count**	**frequency**	**z**
silver	*silver*	approach → gonopodial extension	31	0.007	6.37
		approach → orient	416	0.089	93.95
		follow → approach	53	0.011	11.37
		follow → orient	53	0.011	11.37
		frontal display → approach	47	0.010	10.01
		frontal display → move away	7	0.002	0.91
		frontal display → orient	47	0.010	10.01
		gonopodial extension → approach	29	0.006	5.91
		gonopodial extension → orient	37	0.008	7.73
		gonopodial extension → move away	4	0.001	0.23
		lateral display → approach	54	0.012	11.60
		lateral display → frontal display	39	0.008	8.19
		move away → frontal display	27	0.006	5.46
		move away → gonopodial extension	0	0	N/A
		orient → approach	170	0.037	37.99
		orient → chase	50	0.011	10.69
		orient → follow	38	0.008	7.96
		orient → gonopodial extension	88	0.019	19.34
		orient → wiggle	0	0	N/A
		wiggle → orient	0	0	N/A
silver	*mottled*	approach → gonopodial extension	34	0.008	14.56
		approach → orient	94	0.023	41.85
		follow → approach	8	0.002	2.73
		follow → orient	14	0.003	5.46
		frontal display → approach	30	0.007	12.74
		frontal display → move away	41	0.010	17.74
		frontal display → orient	19	0.005	7.73
		gonopodial extension → approach	15	0.004	5.91
		gonopodial extension → orient	14	0.003	5.46
		gonopodial extension → move away	65	0.016	28.66
		lateral display → approach	28	0.007	11.83
		lateral display → frontal display	66	0.016	29.12
		move away → frontal display	48	0.012	20.93
		move away → gonopodial extension	53	0.013	23.20

		orient → approach	47	0.012	20.47
		orient → chase	2	0.000	0.00
		orient → follow	16	0.004	6.37
		orient → gonopodial extension	31	0.008	13.19
		orient → wiggle	1	0.000	-0.45
		wiggle → orient	1	0.000	-0.45
mottled	*mottled*	approach → gonopodial extension	90	0.026	15.74
		approach → orient	157	0.046	27.93
		follow → approach	5	0.001	0.27
		follow → orient	5	0.001	0.27
		frontal display → approach	28	0.008	4.46
		frontal display → move away	1	0.000	-0.45
		frontal display → orient	20	0.006	3.00
		gonopodial extension → approach	61	0.018	10.46
		gonopodial extension → orient	49	0.014	8.28
		gonopodial extension → move away	3	0.001	-0.09
		lateral display → approach	30	0.009	4.82
		lateral display → frontal display	28	0.008	4.46
		move away → frontal display	2	0.001	-0.27
		move away → gonopodial extension	3	0.001	-0.09
		orient → approach	149	0.044	26.48
		orient → chase	0	0	N/A
		orient → follow	6	0.002	0.45
		orient → gonopodial extension	68	0.020	11.74
		orient → wiggle	191	0.056	34.12
		wiggle → orient	191	0.056	34.12
mottled	*silver*	approach → gonopodial extension	81	0.014	11.68
		approach → orient	563	0.100	84.77
		follow → approach	98	0.017	14.25
		follow → orient	229	0.041	34.12
		frontal display → approach	89	0.016	12.89
		frontal display → move away	7	0.001	0.45
		frontal display → orient	37	0.007	5.00
		gonopodial extension → approach	63	0.011	8.95
		gonopodial extension → orient	70	0.012	10.01
		gonopodial extension → move away	9	0.002	0.76
		lateral display → approach	43	0.008	5.91
		lateral display → frontal display	58	0.010	8.19
		move away → frontal display	13	0.002	1.36

move away → gonopodial extension	7	0.001	0.45
orient → approach	368	0.066	55.20
orient → chase	19	0.003	2.27
orient → follow	190	0.034	28.21
orient → gonopodial extension	153	0.027	22.60
orient → wiggle	9	0.002	0.76
wiggle → orient	12	0.002	1.21

Table S17. Comparison of social behavior transitions used by male mosquitofish morphs towards same- or different-color males in dyadic encounters. Behavioral transitions that contributed significantly to overall social behavioral sequence are compared using Kruskal-Wallis tests. Significant differences are highlighted in bold for P-values below the Bonferroni-adjusted alpha (0.0025). The transitions *follow → orient* and *move away → gonopodial extension* differ for at least one dyad compared to the others.

transition	chi-squared	df	P-value
approach → gonopodial extension	3.76	3	0.289
approach → orient	13.43	3	0.004
follow → approach	13.36	3	0.004
follow → orient	**15.80**	**3**	**0.001**
frontal display → approach	5.41	3	0.144
frontal display → move away	10.93	3	0.012
frontal display → orient	0.93	3	0.819
gonopodial extension → approach	2.04	3	0.564
gonopodial extension → move away	9.64	3	0.022
gonopodial extension → orient	6.08	3	0.108
lateral display → approach	2.23	3	0.527
lateral display → frontal display	4.43	3	0.219
move away → frontal display	10.31	3	0.016
move away → gonopodial extension	**16.33**	**3**	**0.001**
orient → approach	11.77	3	0.008
orient → chase	11.84	3	0.008
orient → follow	9.16	3	0.027
orient → gonopodial extension	3.06	3	0.383
orient → wiggle	1.08	3	0.783
wiggle → orient	1.08	3	0.783

Table S18. Contrasts of significantly different behavioral transitions for male mosquitofish morphs towards same- or different-color males in dyadic encounters. Contrasts were compared with Wilcoxon-ranked pairwise *post-hoc* tests. Social context is indicated as focal male > stimulus male. Males are either mottled (m) or silver (s). Significant differences are highlighted in bold for Bonferroni-adjusted P-values. Silver males use the transition *follow → orient* with silver males more than mottled males. Silver males use more *move away → gonopodial extension* with mottled males than with silver males.

transition	contrast 1	contrast 2	P-value
follow → orient	m > s	m > m	0.13
	s > m	m > m	1.00
	s > s	**m > m**	**0.01**
	s > m	m > s	0.08
	s > s	m > s	1.00
	s > s	**s > m**	**0.03**
move away → gonopodial extension	m > s	m > m	1.00
	s > m	m > m	0.11
	s > s	m > m	1.00
	s > m	**m > s**	**0.04**
	s > s	m > s	1.00
	s > s	**s > m**	**0.01**

Table S19. Transitions that contribute significantly to the social behavior sequence used by each male mosquitofish morph with same-color or different-color males, as well as females, in mix-sex contexts. Behaviors are directed towards a female (f), a mottled male (m), or a silver male (s). Counts are pooled across trials. Frequencies are calculated based on all possible transitions. Z-scores are calculated based median absolute deviation for transitions with frequencies > 0. Only transitions with P-values less than the Bonferroni-adjusted alpha for at least one morph are included here.

focal	**other male**	**transition**	**count**	**frequency**	**z**
silver	*silver*	bite (f) → lateral display (s)	121	0.015	17.74
		bitten (f) → lateral display (s)	121	0.015	17.74
		bitten (f) → lunge (s)	390	0.049	58.54
		chase (f) → move away (s)	67	0.008	9.55
		escape (f) → hiding (s)	490	0.062	73.70
		escape (s) → bitten (f)	96	0.012	13.95
		escape (s) → lunge (s)	52	0.007	7.28
		follow (f) → hiding (s)	128	0.016	18.80
		follow (s) → lateral display (s)	354	0.045	53.08
		frontal display (f) → hiding (s)	128	0.016	18.80
		frontal display (s) → lateral display (s)	354	0.045	53.08
		gonopodial extension (s) → lateral display (s)	93	0.012	13.50
		gonopodial swing (s) → lateral display (s)	93	0.012	13.50
		hiding (s) → "face-to-face" display (s)	101	0.013	14.71
		hiding (s) → bitten (f)	345	0.044	51.71
		hiding (s) → chase (f)	102	0.013	14.86
		hiding (s) → escape (s)	121	0.015	17.74
		hiding (s) → gonopodial extension (s)	30	0.004	3.94
		hiding (s) → lunge (s)	299	0.038	44.74
		hiding (s) → move away (s)	86	0.011	12.44
		hiding (s) → skipping (f)	97	0.012	14.10
		lateral display (f) → lateral display (s)	48	0.006	6.67
		lunge (f) → lateral display (s)	48	0.006	6.67
		lunge (s) → "face-to-face" display (s)	25	0.003	3.18
		lunge (s) → bitten (f)	365	0.046	54.74
		lunge (s) → skipping (f)	149	0.019	21.99
		move away (s) → chase (f)	61	0.008	8.64
		skipping (f) → bitten (f)	140	0.018	20.62
		skipping (f) → lunge (s)	136	0.017	20.02
silver	*mottled*	bite (f) → lateral display (m)	149	0.037	33.21
		bitten (f) → lateral display (m)	149	0.037	33.21
		bitten (f) → lunge (m)	273	0.068	61.42
		chase (f) → move away (m)	0	0	N/A

		escape (f) → hiding (m)	249	0.062	55.96
		escape (m) → bitten (f)	59	0.015	12.74
		escape (m) → lunge (m)	53	0.013	11.37
		follow (f) → hiding (m)	0	0	N/A
		follow (m) → lateral display (m)	321	0.080	72.34
		frontal display (f) → hiding (m)	0	0	N/A
		frontal display (m) → lateral display (m)	321	0.080	72.34
		gonopodial extension (m) → lateral display (m)	0	0	N/A
		gonopodial swing (m) → lateral display (m)	0	0	N/A
		hiding (m) → "face-to-face" display (m)	128	0.032	28.43
		hiding (m) → bitten (f)	218	0.054	48.91
		hiding (m) → chase (f)	0	0	N/A
		hiding (m) → escape (m)	100	0.025	22.06
		hiding (m) → gonopodial extension (m)	58	0.014	12.51
		hiding (m) → lunge (m)	243	0.061	54.59
		hiding (m) → move away (m)	0	0	N/A
		hiding (m) → skipping (f)	21	0.005	4.09
		lateral display (f) → lateral display (m)	51	0.013	10.92
		lunge (f) → lateral display (m)	51	0.013	10.92
		lunge (m) → "face-to-face" display (m)	53	0.013	11.37
		lunge (m) → bitten (f)	239	0.060	53.68
		lunge (m) → skipping (f)	61	0.015	13.19
		move away (m) → chase (f)	0	0	N/A
		skipping (f) → bitten (f)	49	0.012	10.46
		skipping (f) → lunge (m)	41	0.010	8.64
mottled	*mottled*	bite (f) → lateral display (m)	167	0.034	10.33
		bitten (f) → lateral display (m)	167	0.034	10.33
		bitten (f) → lunge (m)	274	0.055	17.29
		chase (f) → move away (m)	0	0	N/A
		escape (f) → hiding (m)	381	0.077	24.24
		escape (m) → bitten (f)	67	0.013	3.83
		escape (m) → lunge (m)	55	0.011	3.05
		follow (f) → hiding (m)	0	0	N/A
		follow (m) → lateral display (m)	292	0.059	18.46
		frontal display (f) → hiding (m)	0	0	N/A
		frontal display (m) → lateral display (m)	292	0.059	18.46
		gonopodial extension (m) → lateral display (m)	0	0	N/A
		gonopodial swing (m) → lateral display (m)	0	0	N/A
		hiding (m) → "face-to-face" display (m)	148	0.030	9.10

		hiding (m) → bitten (f)	272	0.055	17.16
		hiding (m) → chase (f)	0	0	N/A
		hiding (m) → escape (m)	80	0.016	4.68
		hiding (m) → gonopodial extension (m)	1	0.000	-0.45
		hiding (m) → lunge (m)	280	0.056	17.68
		hiding (m) → move away (m)	0	0	N/A
		hiding (m) → skipping (f)	86	0.017	5.07
		lateral display (f) → lateral display (m)	30	0.006	1.43
		lunge (f) → lateral display (m)	30	0.006	1.43
		lunge (m) → “face-to-face” display (m)	23	0.005	0.97
		lunge (m) → bitten (f)	254	0.051	15.99
		lunge (m) → skipping (f)	96	0.019	5.72
		move away (m) → chase (f)	0	0	N/A
		skipping (f) → bitten (f)	102	0.021	6.11
		skipping (f) → lunge (m)	75	0.015	4.35
mottled	*silver*	bite (f) → lateral display (s)	188	0.023	27.90
		bitten (f) → lateral display (s)	188	0.023	27.90
		bitten (f) → lunge (s)	566	0.069	85.23
		chase (f) → move away (s)	142	0.017	20.93
		escape (f) → hiding (s)	379	0.046	56.87
		escape (s) → bitten (f)	71	0.009	10.16
		escape (s) → lunge (s)	59	0.007	8.34
		follow (f) → hiding (s)	91	0.011	13.19
		follow (s) → lateral display (s)	410	0.050	61.57
		frontal display (f) → hiding (s)	91	0.011	13.19
		frontal display (s) → lateral display (s)	410	0.050	61.57
		gonopodial extension (s) → lateral display (s)	99	0.012	14.41
		gonopodial swing (s) → lateral display (s)	99	0.012	14.41
		hiding (s) → "face-to-face" display (s)	156	0.019	23.05
		hiding (s) → bitten (f)	293	0.036	43.83
		hiding (s) → chase (f)	59	0.007	8.34
		hiding (s) → escape (s)	59	0.007	8.34
		hiding (s) → gonopodial extension (s)	0	0	N/A
		hiding (s) → lunge (s)	386	0.047	57.93
		hiding (s) → move away (s)	96	0.012	13.95
		hiding (s) → skipping (f)	99	0.012	14.41
		lateral display (f) → lateral display (s)	14	0.002	1.52
		lunge (f) → lateral display (s)	14	0.002	1.52
		lunge (s) → “face-to-face” display (s)	72	0.009	10.31

	lunge (s) → bitten (f)	462	0.057	69.45
	lunge (s) → skipping (f)	278	0.034	41.55
	move away (s) → chase (f)	101	0.012	14.71
	skipping (f) → bitten (f)	209	0.026	31.09
	skipping (f) → lunge (s)	215	0.026	32.00

Table S20. Comparison of social behavior transitions used by male mosquitofish morphs with same-color or different-color males, as well as females, in mix-sex contexts. Behavioral transitions that contributed significantly to overall social behavioral sequence were compared using Kruskal-Wallis tests. Behaviors are directed towards a female (f) or a male (m). Results of the Kruskal-Wallis tests are presented. Significant differences are highlighted in bold for P-values below the Bonferroni-adjusted alpha (0.001). Some transitions differed for at least one context. Three transitions are from female-directed assertive behaviors to male-directed submissive behaviors and two transitions are from male-directed assertive behaviors to female-directed submissive behaviors. For behavioral transitions only between male-directed behaviors, two transitions were between assertive behaviors while one transition was between submissive behaviors.

transition	**chi-squared**	**df**	**P-value**
bite (f) → lateral display (m)	1.49	3	0.684
bitten (f) → lateral display (m)	1.49	3	0.684
bitten (f) → lunge (m)	4.06	3	0.255
chase (f) → move away (m)	**24.17**	**3**	**<0.001**
escape (f) → hiding (m)	3.33	3	0.344
escape (m) → bitten (f)	4.17	3	0.244
escape (m) → lunge (m)	3.32	3	0.345
follow (f) → hiding (m)	**22.91**	**3**	**<0.001**
follow (m) → lateral display (m)	3.94	3	0.268
frontal display (f) → hiding (m)	**22.91**	**3**	**<0.001**
frontal display (m) → lateral display (m)	3.94	3	0.268
gonopodial extension (m) → lateral display (m)	21.71	3	**<0.001**
gonopodial swing (m) → lateral display (m)	**21.71**	**3**	**<0.001**
hiding (m) → "face-to-face" display (m)	2.03	3	0.566
hiding (m) → bitten (f)	4.42	3	0.219
hiding (m) → chase (f)	**26.98**	**3**	**<0.001**
hiding (m) → escape (m)	8.99	3	0.029
hiding (m) → gonopodial extension (m)	12.05	3	0.007
hiding (m) → lunge (m)	3.02	3	0.389
hiding (m) → move away (m)	**24.05**	**3**	**<0.001**
hiding (m) → skipping (f)	3.54	3	0.315
lateral display (f) → lateral display (m)	7.11	3	0.069
lunge (f) → lateral display (m)	7.11	3	0.069
lunge (m) → "face-to-face" display (m)	1.98	3	0.577
lunge (m) → bitten (f)	3.43	3	0.330
lunge (m) → skipping (f)	1.39	3	0.708
move away (m) → chase (f)	**24.11**	**3**	**<0.001**
skipping (f) → bitten (f)	2.30	3	0.513
skipping (f) → lunge (m)	3.10	3	0.377

Table S21. Contrasts of significantly different behavioral transitions for male mosquitofish morphs with same- or different-color males in dyadic encounters, as well as females, in mix-sex contexts. Contrasts were compared with Wilcoxon-ranked pairwise *post-hoc* tests. Social context is indicated as focal male > stimulus male (observer females). Fish identity is noted by female (f), mottled (m), or silver (s). Significant differences are highlighted in bold for Bonferroni-adjusted P-values. Both morphs used a subset of transitions in contexts with silver males that they did not in contexts with mottled males.

transition	**contrast 1**	**contrast 2**	**P-value**
chase (f) → move away (m)	**m > s (f)**	**m > m (f)**	**0.007**
	s > s (f)	**m > m (f)**	**0.002**
	s > m (f)	**m > s (f)**	**0.007**
	s > s (f)	m > s (f)	1.000
	s > s (f)	**s > m (f)**	**0.002**
follow (f) → hiding (m)	**m > s (f)**	**m > m (f)**	**0.023**
	s > s (f)	**m > m (f)**	**0.002**
	s > m (f)	**m > s (f)**	**0.023**
	s > s (f)	m > s (f)	1.000
	s > s (f)	**s > m (f)**	**0.002**
frontal display (f) → hiding (m)	**m > s (f)**	**m > m (f)**	**0.023**
	s > s (f)	**m > m (f)**	**0.002**
	s > m (f)	**m > s (f)**	**0.023**
	s > s (f)	m > s (f)	1.000
	s > s (f)	**s > m (f)**	**0.002**
gonopodial extension (m) → lateral display (m)	**m > s (f)**	**m > m (f)**	**0.007**
	s > s (f)	**m > m (f)**	**0.007**
	s > m (f)	**m > s (f)**	**0.007**
	s > s (f)	m > s (f)	1.000
	s > s (f)	**s > m (f)**	**0.007**
gonopodial swing (m) → lateral display (m)	**m > s (f)**	**m > m (f)**	**0.007**
	s > s (f)	**m > m (f)**	**0.007**
	s > m (f)	**m > s (f)**	**0.007**
	s > s (f)	m > s (f)	1.000
	s > s (f)	**s > m (f)**	**0.007**
hiding (m) → chase (f)	**m > s (f)**	**m > m (f)**	**0.002**
	s > s (f)	**m > m (f)**	**0.002**
	s > m (f)	**m > s (f)**	**0.002**
	s > s (f)	m > s (f)	1.000
	s > s (f)	**s > m (f)**	**0.002**

hiding (m) → move away (m)	**m > s (f)**	**m > m (f)**	**0.007**
	s > s (f)	**m > m (f)**	**0.002**
	s > m (f)	**m > s (f)**	**0.007**
	s > s (f)	m > s (f)	1.000
	s > s (f)	**s > m (f)**	**0.002**
move away (m) → chase (f)	**m > s (f)**	**m > m (f)**	**0.007**
	s > s (f)	**m > m (f)**	**0.002**
	s > m (f)	**m > s (f)**	**0.007**
	s > s (f)	m > s (f)	1.000
	s > s (f)	**s > m (f)**	**0.002**

CHAPTER 4

Social Conflict Outcomes in Male Eastern Mosquitofish (*Gambusia holbrooki*) Color Morphs Depends on Size and Morph

4.1 Abstract

Most male Eastern Mosquitofish (*Gambusia holbrooki*) are silver, but a few males express a mottled pattern. Silver mosquitofish exhibit size-dependent dominance, in which larger males dominate smaller males; however, it is unknown whether mottled males also use size to determine social dominance. Mottled males were previously found to dominate silver males in size-matched dyads and in mix-sex groups (*Chapter 3*), but whether this dominance advantage is maintained across a range of size differences is unknown. I investigated how both opponent size and morph affected outcomes of social conflict. I recorded aggressive and submissive behaviors for male dyads of every color morph-combination across a range of size differences and then calculated a dominance index that defined social conflict outcome. Larger relative body size predicted dominance when males fought same-color opponents, but morph was more important than size in conflicts with opponents of the opposite morph. Mottled males had a higher probability of dominating silver males over a range of size differences even when they were smaller. Dominant males of the two morphs differed in their behaviors, depending on their opponents' morph. Among subordinate males, only silver males changed their behaviors based on opponent morph. Silver males decreased their use of assertive behaviors with mottled opponents relative to silver opponents. Generally, dominant males showed more assertive and less submissive behaviors than subordinate males; however, in the uncommon situation when a silver male dominated a mottled opponent, subordinate

mottled males were not significantly less assertive, which may explain why silver males so infrequently dominate mottled males. In conclusion, both morphs respond differently to opponent males of different sizes and color morph, which then determines social conflict outcomes.

4.2 Introduction

Within populations, individuals can have different suites of correlated morphological, physiological, and behavioral traits, resulting in alternative phenotypes (rev: Brockmann, 2001; Ducrest *et al*., 2008; McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018). Social interactions depend on the expressed traits of both focal and conspecific interacting individuals (e.g., body size, color pattern, social behavior; Moore *et al*., 1997; Wolf *et al*., 1998; McGlothlin *et al*., 2010; Wilson *et al*., 2011). Social interaction outcomes among alternative phenotypes, such as social dominance, are not just dependent on direct genetic differences between morphs, but also the indirect genetic effects of the phenotypes expressed by interacting individuals (Moore *et al*., 1997; Wolf and Weissing, 2010; Wilson *et al*., 2011).

Social dominance correlates with melanin-based color polymorphisms across taxa (rev: Ducrest *et al*., 2008; San-Jose and Roulin, 2018). Generally, darker morphs are dominant over lighter morphs in insects (e.g., Tibbets and Dale, 2004), fish (e.g., Johnson and Fuller, 2015), reptiles (e.g., Bruinjé *et al*., 2019), birds (e.g., Rohwer, 1975; Rohwer and Ewald, 1981; Mennill *et al*., 2003; Nakagawa *et al*., 2007; Rat *et al*., 2015), and mammals (e.g., Loehr *et al*., 2008). Eastern Mosquitofish (*Gambusia holbrooki*) males exhibit a discrete color polymorphism. Males are commonly unspotted (silver), but a low frequency of males express irregularly distributed black blotches on their bodies (mottled, but previously referred to as melanic or melanistic; first described by Myers, 1925; Regan, 1961; Angus, 1989; Horth, 2006; rev: Zerulla and Stoddard, 2021). Male mosquitofish morphs are an amenable system for studying social dominance differences between alternative phenotypes. Mosquitofish are social species that often aggregate in groups (Itzkowitz, 1971; Pyke, 2005), so dominance hierarchies form often; these hierarchies are known to be extremely stable for species in the genus *Gambusia* (Caldwell and Caldwell, 1962).

In both dyads and mix-sex contexts, size-matched mottled males dominate silver males; this dominance is driven by silver males depressing their assertive behaviors and increasing their submissive behaviors towards mottled males compared to silver males (*Chapter 3*; Horth, 2003; Kraft *et al*., 2018). Color morph (i.e., the color pattern and its correlated behavioral traits) may determine social conflict outcomes for size-matched mosquitofish males, but whether these social dominance differences are maintained across the range of size differences in wild populations is not known. Because size predicts dominance for silver male *G. holbrooki* (Matthews and Wong, 2015), size may have an important indirect genetic effect on social conflict outcomes for male mosquitofish morphs. I investigated how both morph and size affect social conflict outcomes for within- and between-morph interactions of male Eastern Mosquitofish.

Because size predicted dominance rank for silver male *G. holbrooki* (Matthews and Wong, 2015), I predicted that larger silver males would dominate smaller silver males. If size is important in determining social conflict outcomes between mottled males, larger mottled males should dominate smaller mottled males. In this case, mottled males may use size as a cue to alter social dominance, or larger males may just have the greater reserves required to win a war of attrition against smaller males. If size does not affect within-morph social conflict outcomes for mottled males, which has been observed in another poecilid, *Heterandria formosa* (Bisazza *et al*., 1996), then they will have an equal probability of becoming dominant regardless of a size difference.

If size affects social conflict outcomes in between-morph interactions, mottled males may dominate silver males when size-matched, but larger silver males would dominate smaller mottled males once they achieve a certain threshold in relative size difference. If size is not important, then morph is hypothesized to be the primary determinant of social conflict outcomes in between-morph interactions. Mottled males would be predicted to dominate silver males regardless of size differences, so smaller mottled males would dominate larger silver males. In this case, color pattern and associated dominant and subordinate behavioral strategies may be more important in predicting conflict outcomes, such as in Aegean wall lizards, *Podarxis erardii* (Brock *et al*., 2022). I also investigated whether social dominance behaviors differ between male mosquitofish morphs based on social status and opponent morph.

4.3 Methods

4.3.1 Experimental Fish

Wild Eastern Mosquitofish (*G. holbrooki*) were collected by dip-netting from the Student Housing Pond (SHP: 25.757011°N, 80.370483°W) on the MMC campus of Florida International University in Miami, Florida. After capture, male standard length (tip of the snout to the end of the caudal peduncle) was briefly measured using a caliper. Two size-matched males of each color morph were selected for five length classes: 15-19, 20-22, 24-25, 26-28, and 29-30 mm. If a male died, he was immediately replaced by a size-matched male of the same morph. Males were then housed individually in 19 L aquaria (40 cm long x 25 cm high x 21 cm deep) with crushed limestone pea gravel on the bottom. Individual aquaria were set within larger tubs that held females that swam among the male aquaria as social stimuli (10 single male aquaria per tub surrounded by 10 females). Males interacted with these females by swimming against the glass; however, male aquaria were spaced apart in a way that they were not observed to be interacting with each other. The light cycle was 12:12 and the water temperature was 22°C. I previously determined that individual fish do not eat more than 0.01 g of TetraColor Tropical Flakes (Tetra) per day, so fish were given 0.01g of fish flakes at the end of the day and any leftover food was removed from their aquaria in the morning.

Silver males were freeze-branded using liquid nitrogen to identify individuals in video recordings. Males were placed in a 1.9-liter plastic holding aquarium. They were lifted for a few seconds in a net to brand a small spot on either the right or left side. Fish were immersed in water containing Stress Coat (API) and allowed to recover for 30 min afterwards within the holding aquarium. Silver males were re-branded every month (~30 days) for the duration of the experiment. Following branding, all males exhibited normal swimming. This freeze branding method did not work on mottled male skin; thus, their natural individually distinctive color patterns were used to identify individuals. Mottled males were still handled on the same days as silver males. All lab methods were carried out under the approval of FIU's Institutional Animal Care and Use Committee (IACUC-18-039-CCR01; IACUC-18-039-AE01; IACUC-18-039-CR02; IACUC-19-075; IACUC-20-035).

4.3.2 Length and Melanism Analysis

Since fish may grow longer during the course of the study, fish size was estimated with *ImageJ* software (v.1.52k; Schneider *et al*., 2012) using images captured from the video for each trial. The scale was calibrated using known distances in the aquarium. For each fish, the standard length (tip of the snout to the end of the caudal peduncle) was measured twice and then averaged. The video-based measurements taken in the behavioral arena were corrected based on 127 measurements comparing this protocol and their caliper measurements: 0.9348 * mean video-based length + 1.103 mm. These sizes were confirmed against weekly caliper measurements. Relative size difference between males was calculated as [focal male length – stimulus male length] / [focal male length]. Images from both sides of the fish were taken from the video. The fish's body was outlined in Adobe *Fresco* (2022), and then all black spots were traced and filled-in. *ImageJ* calculated the proportion of black spots present on the total body area of mottled males. Relative color difference between males was calculated based on the proportion of silver present on the total body area: [(1 – focal male melanism) – (1 – opponent male melanism)] / [(1 – focal male melanism)].

4.3.3 Social Conflict Experiment

The behavioral arena was a 19 L aquarium (40 cm long x 25 cm high x 21 cm deep) with limestone pea gravel on the bottom. Two fluorescent lights positioned on either side illuminated the aquarium, but also resulted in a mirror-like reflection on the aquarium's sides. White translucent plastic binder dividers were placed inside against the glass to prevent males from seeing and interacting preferentially with their own reflection. A white foam board was also placed on the aquarium's back to provide contrast for observing the males on video. A video camera (GoPro Hero4, 1080p, 60 fps) was placed on a stand in front of the aquarium. After recording began, the entire set-up was covered with black cloth on all sides to prevent disturbance to fish behavior.

I created a "round robin" match-up design, in which all 20 males interacted with every other male in dyads (n = 190 dyads or 380 focal males; 90 silver-silver, 90 mottled-mottled; 100 silver-mottled). Dyads were randomized across days so that no male was tested more than once per day, and each day had both same-color and different-color male dyads. Fish acclimated in 1.9-liter plastic holding aquaria for 5 min prior to testing and both males of a dyad were then introduced to the aquaria at the same time to prevent time-based dominance. Interactions were recorded for 30 min, after which males were returned to their individual aquaria. Water was replaced between behavioral tests to maintain the temperature at 22°C and to prevent the possible effect of odorants or other released chemicals on the next test.

Male social behavior was scored in BORIS (v 7.10.2; Friard and Gamba, 2016) based on the standardized *G. holbrooki* ethogram (*Chapter 3*). Both the frequency and duration of individual behaviors were recorded for state events, while only frequency was recorded for point events. Since scan sampling accurately measures behavior in mosquitofish (*Chapter 3*), behavior was scored for 1-min within a 3-min interval, resulting in 10 scan samples per dyad. Behavior duration (s) was also summed for all behaviors in each category: aggression, submission, and non-social. A dominance index was calculated for each male: [duration of focal male aggression] / [duration of focal male aggression + duration of focal male submission]. Dominance index was used as a behavioral response measure as I previously found it to be a robust measure of differences between morphs in both male-male dyads and mix-sex contexts (*Chapter 3*). If the focal male had a higher dominance index than his opponent, then he was considered dominant. If the males had equal dominance indices, the male with a higher duration of aggression was considered dominant.

4.3.4 Statistical Analysis

To model the effect of color morph and relative size difference on social conflict outcome, I first determined whether a model using relative color difference or a model using categorical labels for silver and mottled morphs should be used. The model that best fit my data used focal and opponent color morph as factors, rather than a relative color difference (Table S1, S2). Relative size and color differences were correlated (Spearman's rank correlation test: $\rho = -0.27$, $P<0.001$), but relative color difference was not found to interact significantly with relative size difference to affect social conflict outcome and could therefore be replaced with color morph (Table S1). Thus, size and color morph can be examined as separate fixed effects for this analysis.

I used a generalized linear mixed effects model (GLMM) in which the focal color morph, opponent color morph, and relative size difference were included as fixed effects. Because focal and opponent males were repeated across contexts, individual identities were included as random effects. I used social conflict outcome, i.e., whether the focal male was dominant or subordinate, as the responding variable with a binomial distribution. Contrasts were performed using the Tukey method for comparing a family of four estimates. Modelling and contrasts were performed with the R packages: tidyverse v.2.0.0, lme4 v.1.1-32, AICcmodavg v.2.3-2 , ggeffects v.1.2.0, and emmeans v.1.8.5 (Bates *et al.*, 2015; Lüdecke, 2018; Wickham *et al.*, 2019; Lenth, 2022; Mazerolle, 2023).

I also performed a principal components analysis (PCA) on the frequency and duration of each identified behavior to evaluate their contribution to male mosquitofish social behavior using the R packages: tidyverse v.2.0.0, factoextra v.1.0.7, and ggfortify v.0.4.16 (Tang *et al*., 2016; Wickham *et al*., 2019; Kassambara and Mundt, 2020). To determine whether specific behaviors differed among dominant and subordinate fish across the male-male dyads, I used Bayesian modeling to fit generalized linear mixed effects models (GLMM) in which the relative size difference, focal male color morph, opponent color morph, and social conflict outcome (i.e., dominant or subordinate) were included as fixed effects. Because focal and opponent males were repeated across contexts, both of their identities were included as random effects. I used the first four principal components as the responding variable with skewed normal distributions. The signs for PC1 and PC4 values were reversed so that most aggressive behaviors were positive and submissive behaviors were negative, which was more intuitive for both interpretation and graphical display. Using the posterior predicted probability distribution, contrasts were calculated using median marginal estimates and highest posterior probabilities density (HPD) for the 0.95 credible intervals with a multivariate *t*-distribution adjustment. Contrast comparisons were between morphs across within- and between-morph interactions as well as social status when the relative size difference between males was 0 (i.e., size-matched). Modelling and contrasts were performed with the R packages: rstanarm v.2.21.3, brms v.2.19.0, tidybayes v.3.0.4, and emmeans v.1.8.5 (Bürkner, 2021; Goodrich *et al*., 2022; Lenth, 2022; Kay, 2023).

I analyzed behavioral transitions (e.g., A → B, in which behavior A is followed by behavior B) following a sequential analysis previously described by Egge *et al.* (2011) and Worthington and Swallow (2011). Briefly, I calculated counts of first order behavioral transitions for each trial. These counts were summed for dominant and subordinate males in each male-male dyad (mottled → mottled, mottled → silver, silver → silver, mottled → silver) and used to calculate transition frequencies. Behavioral transitions that did not occur (i.e., frequency = 0) or that transitioned with “non-social” or “exclude” were removed from subsequent analysis. I calculated modified z-scores based on median absolute deviation and Bonferroni-adjusted P-values for the remaining transitions to determine which behavioral transitions significantly contributed to morph behavior in each context. Significantly contributing behavioral transitions with frequencies above 0.01 were presented as kinematic diagrams. Behavioral transitions were compared for dominant and subordinate males across dyads. Frequencies for the significant behavioral transitions were first calculated for each observation (n=380 per transition). These frequencies were compared using Kruskal-Wallis ANOVAs followed by Wilcoxon-signed rank pairwise *post-hoc* tests. Significant differences were determined using Bonferroni-adjusted P-values. All statistical analyses and data visualizations were completed in R (v.4.2.3; R Core Team, 2021) and Adobe *Illustrator* (2023).

4.4 Results

4.4.1 Social Conflict Outcomes

Larger relative body size predicts dominance in within-morph dyads, but color morph is more important than size in determining dominance in silver-mottled dyads (Fig. 1; Table 1, S3-4). In within-morph interactions, larger males dominate smaller males for both mottled and silver morphs (Fig. 1; Table 1, S3-4). In silver-mottled interactions, mottled males have a significantly higher probability of being dominant over silver males across a wide range of size differences (Fig. 1; Table 1, S3-4). Although smaller mottled males have a higher probability of achieving dominance over larger silver males (probability: 0.75±0.10), this probability is lower relative to encounters where they were size-matched (probability: 0.91±0.04) or larger (probability: 0.97±0.02) than their opponents.

Table 1. Generalized linear mixed effects model (GLMM) for social conflict outcome in male mosquitofish morph dyads across a range of relative size differences. Male color morph is defined in parentheses: silver (s) and mottled (m). The Akaike Information Criterion (AIC) was 350.4 and the degrees of freedoms for the residuals was 370. Significant effects are highlighted in bold. Dominance is predicted in dyads where the focal male is larger or mottled, or the opponent is a silver male.

Formula: social conflict outcome ~ relative size difference * focal color morph * opponent color + (1 | focal ID) + (1 | opponent ID)

Random	**variance**	**standard deviation**		
focal ID	0.43	0.65		
opponent ID	0.48	0.69		
Fixed	**estimate**	**standard error**	**z**	**P**
intercept	0.09	0.39	0.24	0.81
relative size difference	**5.40**	**1.65**	**3.28**	**0.001**
focal color morph (s)	**-2.30**	**0.54**	**-4.25**	**<0.001**
opponent color morph (s)	**2.20**	**0.54**	**4.05**	**<0.001**
relative size difference * focal color morph (s)	0.49	2.47	0.20	0.84
relative size difference * opponent color morph (s)	-1.40	2.08	-0.67	0.50
focal color morph (s) * opponent color morph (s)	0.11	0.64	0.17	0.86
relative size difference * focal color morph (s) * opponent color morph (s)	3.45	3.23	1.07	0.29

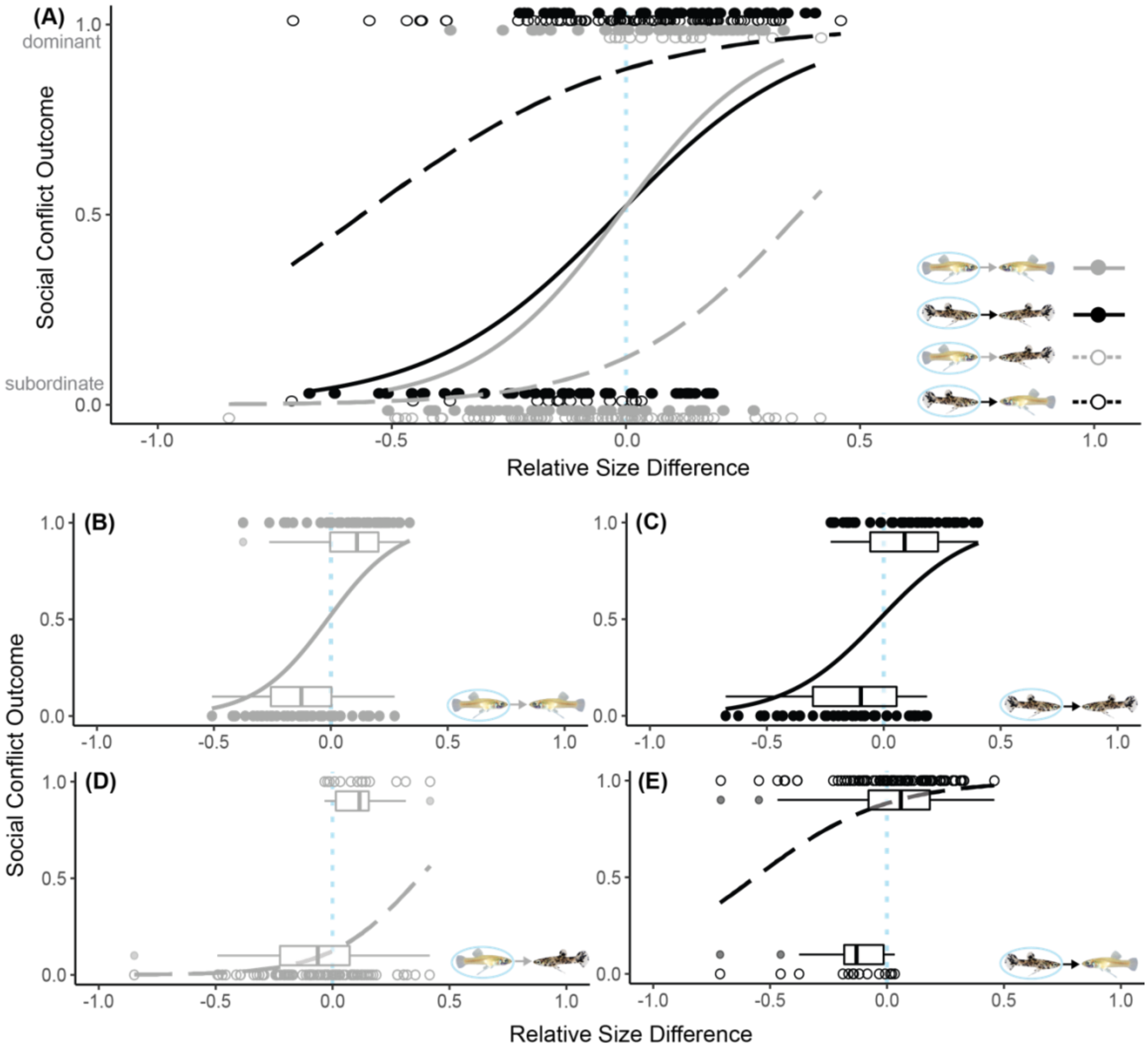

Figure 1. Social conflict outcomes for dyads of male Eastern Mosquitofish (*Gambusia holbrooki*) morphs across a range of relative size differences. Data are fitted with logistic regression lines. Focal males are either silver (gray) or mottled (m). Opponents are either the same (solid line, closed circle) or different (dashed line, open circle) color morphs. Social conflict outcome is represented as the probability of being dominant (1.0) or subordinate (0.0). For the presentation of social conflict outcomes for all dyads **(A)**, points but not regression lines have been slightly moved in position vertically to better see them. Social conflict outcomes are also presented with Tukey-boxplots of the relative size difference of dominant and subordinate males for silver-silver **(B)** and mottled-mottled **(C)** dyads, as well as for focal silver **(D)** and focal mottled **(E)** males in silver-mottled dyads. Larger relative size predicts dominance in within-morph dyads, but morph predicts dominance in silver-mottled dyads. Mottled males dominate silver males across a wide range of relative size differences.

4.4.2 Social Dominance Behaviors

PCA clusters the behaviors into four principal components (PCs) that explain 48% of the variance in the data: 18%, 14%, 10%, 6% (Fig. S1). Based on comparison of loadings and contributions (Fig. S1-2; Table S5), the behaviors that uniquely correlated with each PC could be determined. Assertive behaviors correlate with PC1 (orient, approach, follow, “face-to-face” display), PC3 (swing, circling), and PC4 (cut-off, wiggle). The submissive behavior of moving away correlates with PC2. These PCs are used to examine differences in the use of social dominance behaviors between morphs based on their social status and the opponent’s color morph (Fig. 2; Tables S6-S13).

Dominant males exhibit differences in social behavior expression that depend on both their own morph and that of their opponent. Dominant males of both morphs have a higher PC1 with silver opponents than with mottled opponents (Fig. 2A; Tables S6-7), meaning that they both use more assertive behaviors (orient, approach, follow, and “face-to-face” displays) with silver than mottled opponents. However, dominant silver males use these assertive behaviors more in within-morph conflicts than dominant males do in any other dyad type (Fig. 2A; Tables S6-7); thus, morphs differ in the extent to which they alter these assertive behaviors. Dominant silver males with mottled males also have a higher PC3 (swings, circling) than with silver males and a higher PC3 than mottled males with mottled males (Fig. 2C; Tables S10-11). The few silver males that dominate mottled males use more gonopodial swings and circling, which is a morph-specific response to opponent color morph. Finally, in within-morph interactions, dominant silver males use the behavioral transition *approach → frontal display* significantly more than dominant mottled males (Fig. 3; Tables S14-16), which is another morph-specific response by dominant silver males that is specific to opponent color morph.

Subordinates of the two morphs differ in use of specific social behaviors (Fig. 2; Tables S6-13), but not in behavioral sequence (Fig. 4; Tables S17-18). Subordinate silver males have a lower PC1 (assertive behaviors) with mottled males than with silver males (Fig. 2A; Tables S6-7). They use fewer orients, approaches, follows, and "face-to-face" displays in conflicts with mottled males than in conflicts with other silver males. When interacting with mottled males, subordinate silver males have a higher PC1 and lower PC4 than subordinate mottled males (Fig. 2A, 2E; Tables S6-7, S12-13), meaning that they use the assertive behaviors orient, approach, follow, and "face-to-face" display more, but cut-off and wiggle less. Therefore, subordinate silver males exhibit a morph-specific behavioral response that likewise depends on opponent color morph.

In within-morph conflicts, dominant males have a higher PC1, and thus display more assertive behaviors, than subordinate males (Fig. 2A; Tables S6-7). In between-morph conflicts, dominant mottled males have a higher PC1 (more assertive) than subordinate silver males, but dominant silver males do not differ from subordinate mottled males (Fig. 2A; tables S6-S7). Thus, dominant silver males do not appear to be achieving a strong difference in expression of social dominance behaviors in silver-mottled conflicts. Regardless of focal or opponent color morph, subordinate males are always moving away from opponents more than dominant males (Fig. 2b; Tables S8-9).

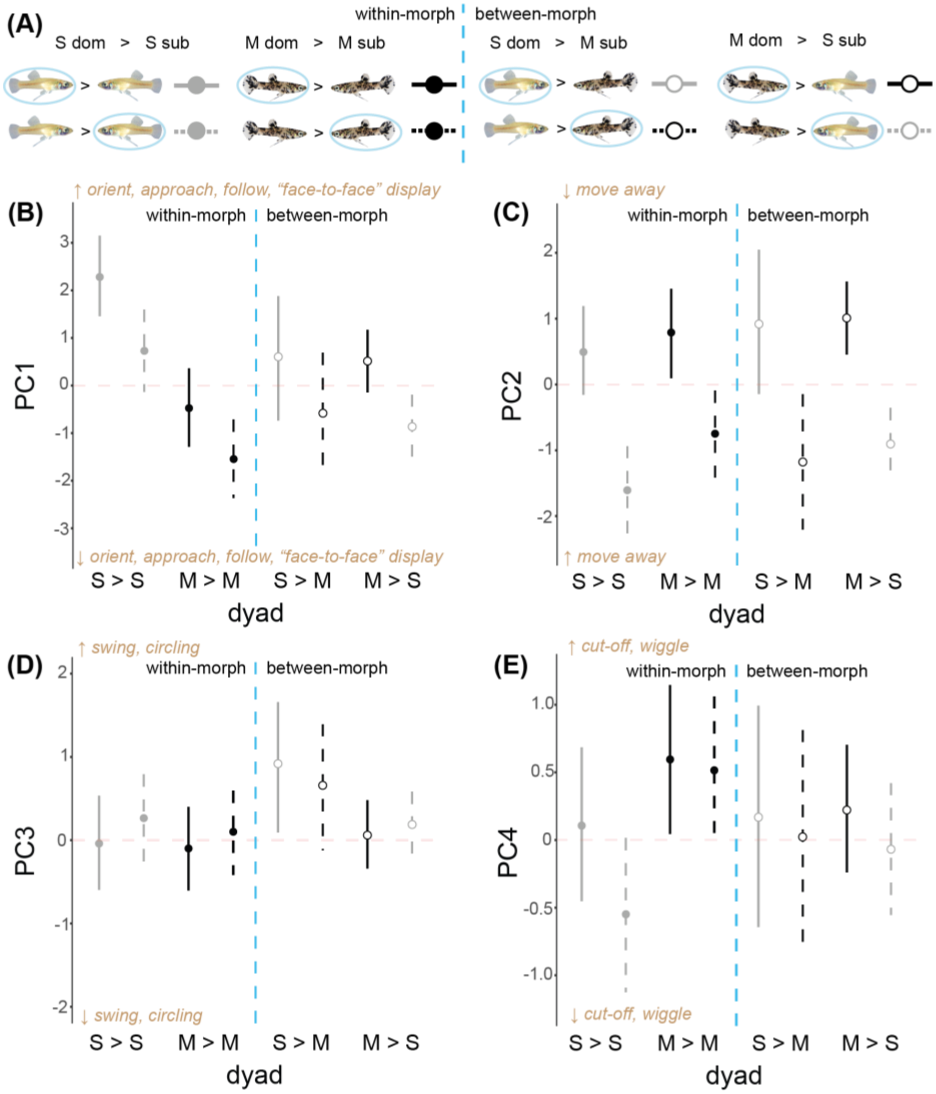

Figure 2. Expression of specific behaviors by dominant and subordinate male mosquitofish in within- and between-morph dyads. Marginal median estimates for the first four PCs at a relative size difference of 0 i.e., size-matched) are shown. Lines represent 0.95 credible intervals based on highest posterior probability densities. The red dashed line at 0 is used to demarcate between higher and lower expression of behaviors. **(A)** Legend. Dyad labels are dominant morph > subordinate morph. Color indicates focal male morph (silver = gray, mottled = black). Closed circles are within-morph interactions, while open circles are between-morph interactions. Line type indicates the focal male's social status: dominant (solid) or subordinate (dashed). **(B)** A higher PC1 means that the male uses more assertive behaviors (orient, approach, follow, "face-to-face" display). Dominant males of both morphs are more assertive towards silver males than mottled males. Dominant silver males are more assertive in within-morph interactions than any other dyad-type. Generally, dominant males are more assertive than subordinate males, except dominant silver males towards subordinate mottled males. Subordinate silver males are less assertive with mottled males than silver males, and less assertive than subordinate mottled males with mottled males. **(C)** All subordinate males move away more (lower PC2) from dominant males. **(D)** Dominant silver males with subordinate silver males have a higher PC3 (more swings and circling) than with subordinate mottled males, and a higher PC3 than dominant mottled males with subordinate mottled males. **(E)** In interactions with dominant mottled males, subordinate silver males have a lower PC4 (fewer cut-offs and wiggles) than subordinate mottled males.

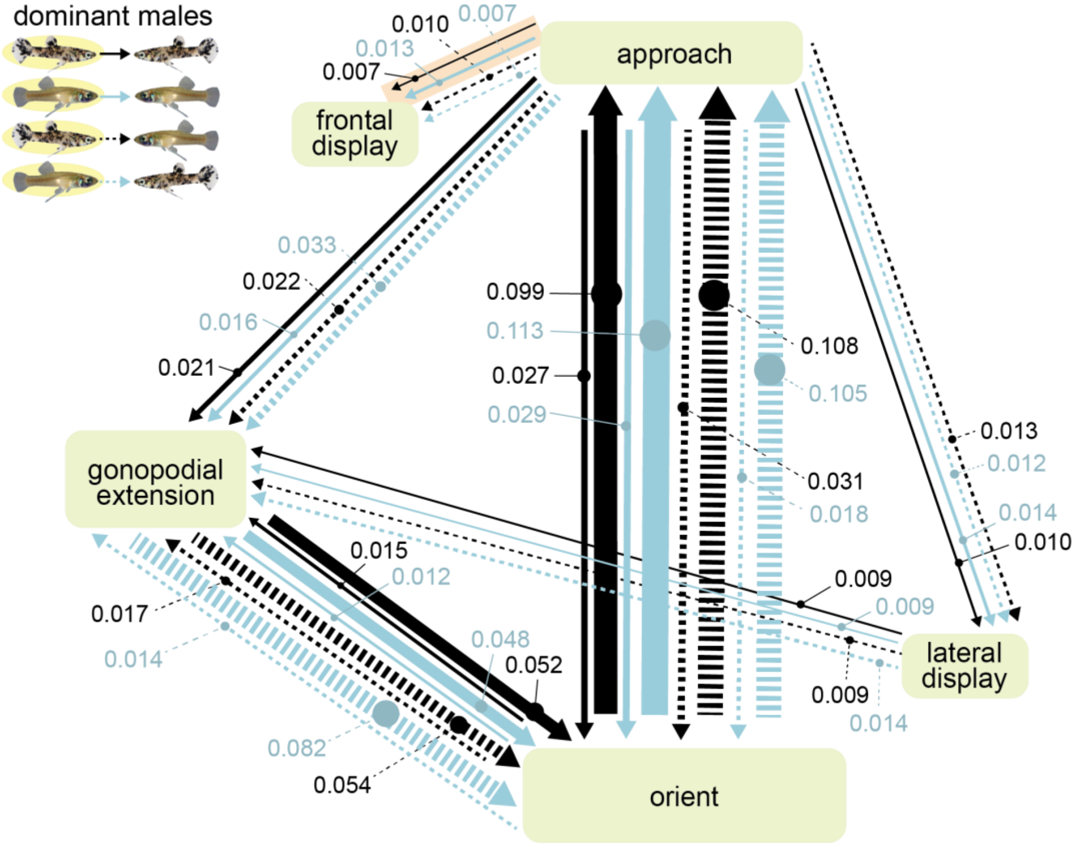

Figure 3. Transitions that significantly contribute to the behavioral sequence of dominant male mosquitofish in within- and between-morph interactions. Frequencies are calculated based on all possible transitions. Arrow weight is proportional to frequency. Mottled male behaviors are black, while silver male behaviors are blue. Line types are solid towards a same-color opponent and dashed towards a different-color opponent. In within-morph interactions, dominant silver males use the behavioral transition *approach* → *frontal display* significantly more than dominant mottled males (highlighted in orange).

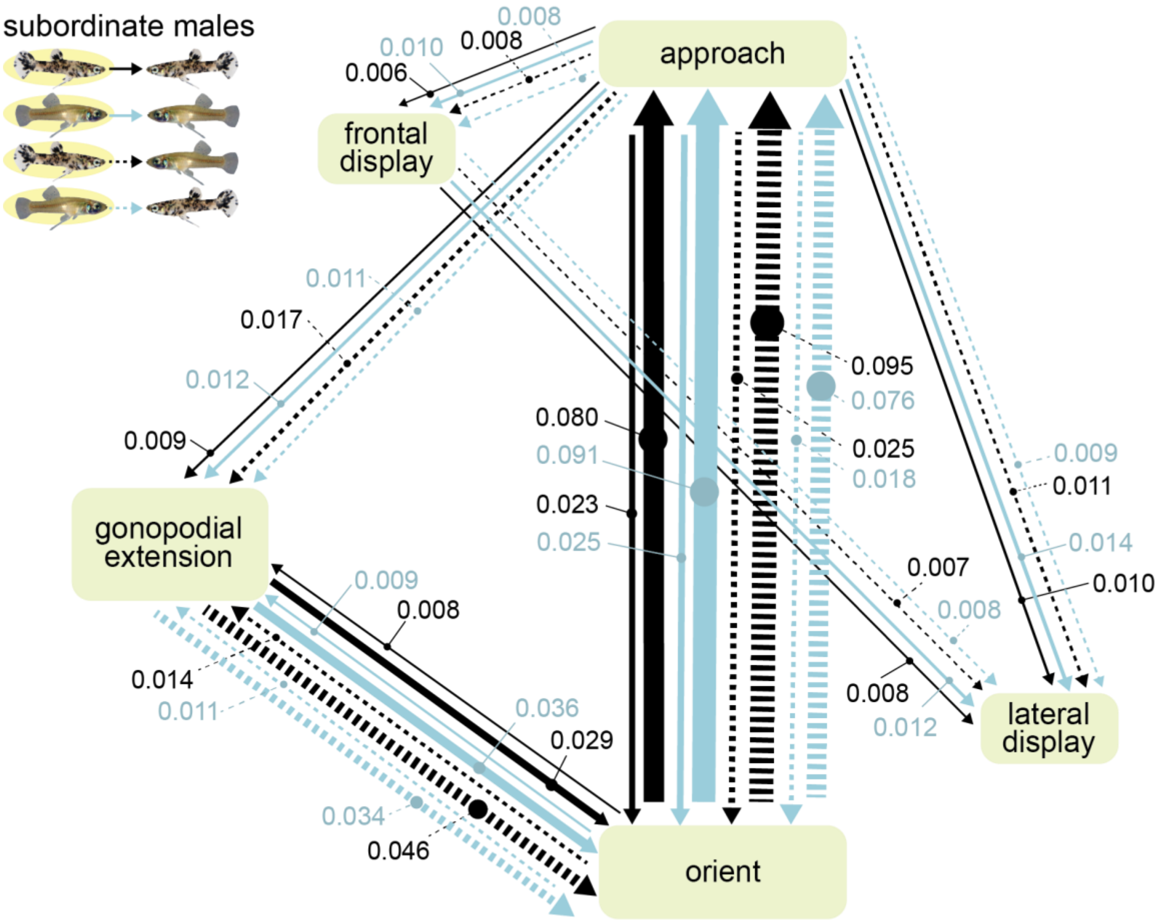

Figure 4. Transitions that significantly contribute to the behavioral sequence of subordinate male mosquitofish in within- and between-morph interactions. Frequencies are calculated based on all possible transitions. Arrow weight is proportional to frequency. Mottled male behaviors are black, while silver male behaviors are blue. Line types are solid towards a same-color opponent and dashed towards a different-color opponent. There are no significant differences in use of behavioral transitions for subordinate male mosquitofish morphs across dyads.

4.5 Discussion

For male *G. holbrooki* color morphs, larger relative body size predicts social dominance in within-morph interactions. However, color morph is a stronger dominance predictor than size in silver-mottled male interactions, with mottled males dominating silver males. In this study, social status and color pattern correlate with differences in the expression of social dominance behaviors; therefore, morphs could be using size, color pattern, dominant and subordinate behavioral strategies, and/or morph-specific patterns in social behavior as cues to guide these social conflict outcomes. Future studies should aim to separate each socially relevant cue from the male mosquitofish morph's behavioral responses. For example, artificially adding black blotches to silver males could test whether mosquitofish males respond to color pattern or morph-specific social behavior. Animations of mosquitofish males could be used to test how size and color pattern alters the expression of social behavior by each morph.

For both morphs, larger males dominate smaller males in within-morph conflicts. This result is in line with previous findings that silver Eastern Mosquitofish have a size-based dominance hierarchy (Matthews and Wong, 2015). Social dominance could increase access to potential mates. The dominant male of a group of silver *G. holbrooki* monopolizes mating attempts on females (Bisazza *et al*., 1996; Dadda *et al*., 2005); however, *G. holbrooki* males that are relatively smaller than females have higher insemination success rates (Pilastro *et al*., 1997). Larger males may dominate female time compared to smaller males of the same morph, but smaller males may have compensatory tactics, so whether size-based dominance provides an advantage that increases reproductive success for this species is unclear. Alternatively, social dominance by larger males could increase their access to other resources, such as foraging sites. Larger size predicts feeding competitive ability and foraging success in fish; however, whether larger size leads to dominance and higher feeding rates or dominance leads to higher feeding rates and larger sizes depends on the system studied (rev: Ward *et al*., 2006).

Both morphs exhibit similar behavioral strategies for social dominance in within-morph interactions. Compared to their opponents, dominant males use more assertive and less submissive behaviors, while subordinate males use less assertive and more submissive behaviors, which matches the pattern previously found in *Gambusia* species (Caldwell and Caldwell, 1962). Nevertheless, some morph-specific behavioral differences appear during within-morph conflicts. Silver males generally use more assertive behaviors than mottled males, and subordinate silver males use less of the behaviors "cut-off" and wiggle compared to subordinate mottled males. Because mosquitofish color patterns have a heritable, genetic basis (Angus, 1989; Horth, 2006), these behavioral differences could be pleiotropically related to color pattern.

Color morph predicts dominance in between-morph conflicts, in which mottled males dominate silver males. These results are similar to previous studies that also found that darker morphs dominate lighter morphs in other taxa (e.g., Rohwer, 1975; Rohwer and Ewald, 1981; Mennill *et al*., 2003; Tibbets and Dale, 2004; Nakagawa *et al*., 2007; Loehr *et al*., 2008; Bruinjé *et al*., 2019). Given that this social dominance is maintained across a wide range of size differences and that Eastern Mosquitofish form stable dominance hierarchies (Caldwell and Caldwell, 1962), social dominance may be part of the selective pressures that stably maintain the mottled morph at a low frequency in mosquitofish populations. Briefly, uncommon morphs are expected to go locally extinct through genetic drift (Wright, 1939; rev: Brockmann, 2001; Brisson, 2018); however, a rare-morph advantage may maintain the polymorphism in a population through negative frequency-dependent selection (Wright, 1939; e.g., Sinervo and Lively, 1996; Dijkstra *et al.*, 2010; rev: Brockmann, 2001; Brisson, 2018). However, whether social dominance even provides a rare-morph selective advantage to mottled males is unknown.

In a previous study, I found no difference between morphs in assertive attention towards females in mix-morph, mix-sex groups (*Chapter 3*), which suggests no difference in access to mates. Sexual selection of morphs by females could also be a stronger determinant of morph reproductive success than social dominance, although female preferences for one morph over the other depends on population, experience, and social environment (rev: Zerulla and Stoddard, 2021). Although juvenile male mosquitofish of the two morphs do not differ in their feeding behavior, they both feed less in the presence of adult mottled males (Culumber *et al.*, 2018). Social dominance of adult mottled males could be driving down feeding in subordinate males, but further study is required. Other factors, such as predation, are likely to exhibit stronger selective pressures on morph equilibrium frequency in mosquitofish populations (Horth, 2004; Horth and Panayotova, 2012; rev: Zerulla and Stoddard, 2021).

In between-morph interactions, mottled males do not adjust their behaviors based on opponent color morph as much as silver males do. The altered behavior of silver males likely leads to mottled males dominating silver males, which is consistent with previous results (*Chapter 3*; Horth, 2003; Kraft *et al.*, 2018). In the rare case that silver males dominate mottled males, silver males do not use significantly more assertive behaviors than their mottled opponents, which would explain why it is difficult for them to achieve dominance in these conflicts. Furthermore, dominant silver males engage in more of an extremely active and aggressive behavior (circling) with subordinate mottled males than they do with subordinate silver males, suggesting that silver males may need to use extreme aggressive acts to dominate mottled males. Silver males also must be much larger than mottled males to increase their probability of achieving dominance. However, in the lab and in the wild, mottled males were larger than silver males (Horth *et al.*, 2010), so unless the mottled morph is a juvenile that is still small and not yet sexually mature, it is unlikely for silver males to dominate mottled males in the wild.

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4.7 Appendix

Table S1. Generalized linear mixed effects model (GLMM) for social conflict outcome across a range of relative size and color differences for male mosquitofish morphs. Male color is defined in parentheses: silver (s) and mottled (m). The Akaike Information Criterion (AIC) was 378 and the degrees of freedoms for the residuals was 374. Significant effects are highlighted in bold. Relative color difference did not significantly interact with relative size difference.

Formula: social conflict outcome ~ relative color difference * relative size difference + (1 | focal ID) + (1 | opponent ID)

Random	**variance**	**standard deviation**
focal ID	0.74	0.86
opponent ID	0.85	0.92

Fixed	**estimate**	**standard error**	**z**	**P**
intercept	-0.15	0.31	-0.48	0.81
relative color difference	**-0.87**	**0.25**	**-3.48**	**0.0005**
relative size difference	**4.24**	**1.14**	**3.72**	**0.0002**
relative color difference * relative size difference	-0.29	1.11	-0.26	0.79

Table S2. AIC comparisons of models that coded color morph as either a continuous or categorical variable. The models being compared are: only random effects (null model), relative color difference as a fixed effect (Table S1), and color morph as fixed effects (Table 1). The GLMM that coded color morph as a categorical variable best fit the data.

model	**K**	**AICc**	**ΔAICc**
color morph	10	351.01	0.00
relative color difference	6	378.09	27.08
random effects only	3	395.00	43.99

Table S3. Marginal estimates for the probability of social dominance of each dyad at three levels of relative size difference in male mosquitofish morphs. Presented comparisons are when the focal male is smaller than his opponent (-0.3), when the focal male is the same size as the opponent (0), and when the focal male is larger than his opponent (+0.3). Dyads are presented as focal morph > opponent morph, where m = mottled and s = silver. Estimates closer to 1.0 indicate dominance, while estimates closer to 0.0 indicate subordinance. Larger focal males have a higher probability of dominance for within-morph conflicts. Mottled males have a higher probability of dominance regardless of relative size differences.

relative size difference	**dyad**	**estimate**	**standard error**
smaller focal male (-0.3)	s > s	0.09	0.06
	m > m	0.18	0.09
	s > m	0.02	0.02
	m > s	0.75	0.10
same size focal male (0)	s > s	0.52	0.10
	m > m	0.52	0.10
	s > m	0.10	0.04
	m > s	0.91	0.04
larger focal male (+0.3)	s > s	0.92	0.05
	m > m	0.84	0.08
	s > m	0.39	0.16
	m > s	0.97	0.02

Table S4. Pairwise contrasts for the probability of social dominance between dyads at three levels of relative size difference in male mosquitofish morphs. Presented comparisons are when the focal male is smaller than his opponent (-0.3), when the focal male is the same size as the opponent (0), and when the focal male is larger than his opponent (+0.3). Marginal effect estimates were calculated for the probability of achieving a dominant social conflict outcome (1.0) and the differences between pairs are presented below with standard error (SE). The Tukey method of P-value adjustment for comparing a family of four estimates was used. Significant results are highlighted in bold. Larger focal males have a higher probability of dominance for within-morph conflicts. Mottled males have a higher probability of dominance regardless of relative size differences.

relative size difference	contrast		difference	SE	z	P
smaller focal male (-0.3)						
	m > m	s > m	0.16	0.09	1.80	0.27
	m > m	**m > s**	**-0.57**	**0.13**	**-4.51**	**<0.0001**
	m > m	s > s	0.09	0.11	0.80	0.86
	s > m	**m > s**	**-0.73**	**0.10**	**-7.00**	**<0.0001**
	s > m	s > s	-0.7	0.06	-1.34	0.60
	m > s	**s > s**	**0.66**	**0.11**	**5.97**	**<0.0001**
same size focal male (0)						
	m > m	**s > m**	**0.42**	**0.10**	**4.48**	**0.0001**
	m > m	**m > s**	**-0.38**	**0.10**	**-3.98**	**0.0004**
	m > m	s > s	-0.003	0.14	-0.02	1.000
	s > m	**m > s**	**-0.81**	**0.06**	**-13.68**	**<0.0001**
	s > m	**s > s**	**-0.43**	**0.10**	**-4.30**	**0.0001**
	m > s	**s > s**	**0.38**	**0.10**	**3.93**	**0.0005**
larger focal male (+0.3)						
	m > m	**s > m**	**0.45**	**0.17**	**2.74**	**0.03**
	m > m	m > s	-0.12	0.08	-1.51	0.43
	m > m	s > s	-0.08	0.10	-0.79	0.86
	s > m	**m > s**	**-0.58**	**0.16**	**-3.62**	**0.002**
	s > m	**s > s**	**-0.53**	**0.16**	**-3.36**	**0.004**
	m > s	s > s	0.047	0.05	0.89	0.81

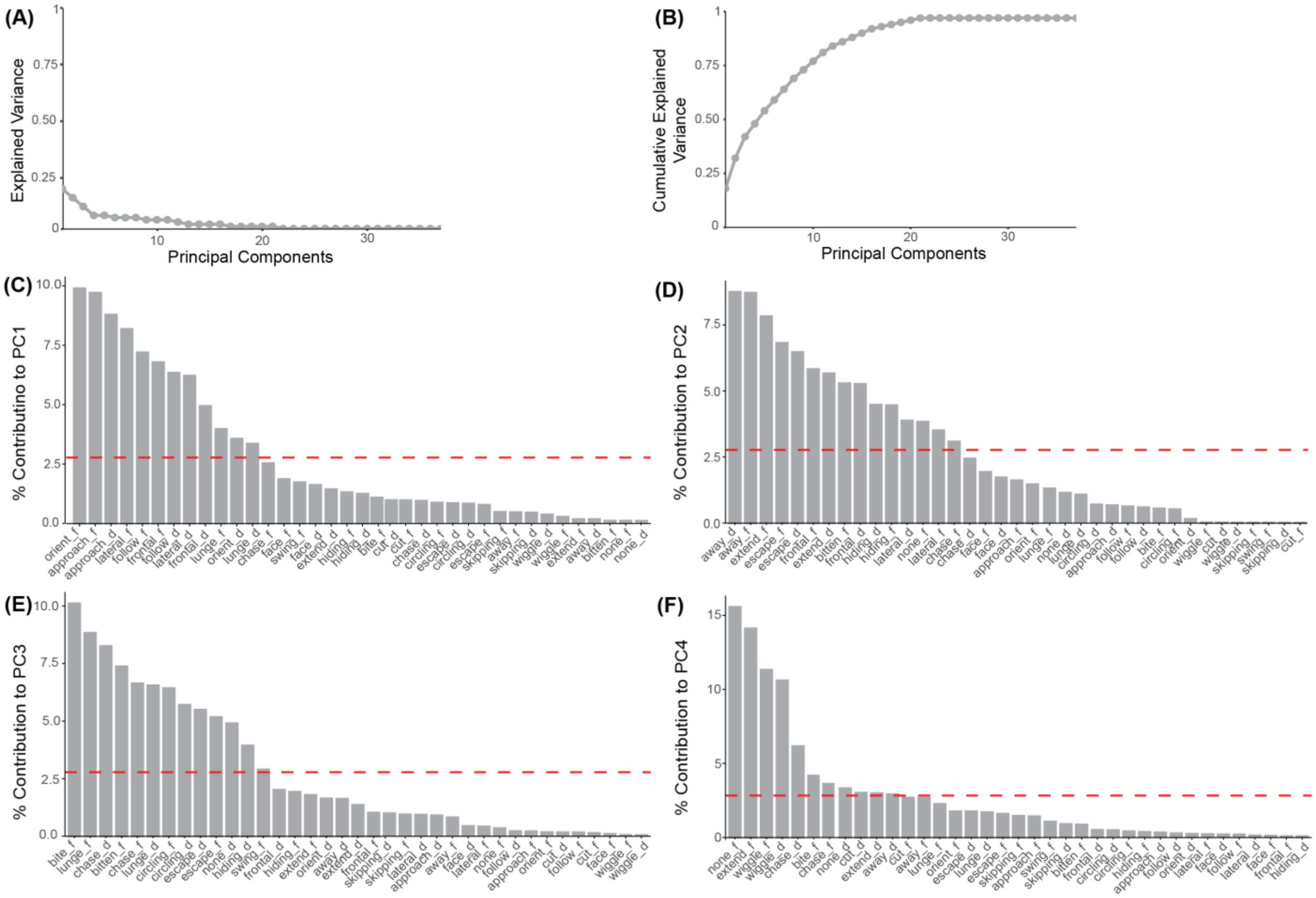

Figure S1. Variance explained by each PC and the percent contribution of behaviors to the first four PCs. Scree plots for the **(A)** explained variance and **(B)** cumulative explained variance of the 37 principal components show that the first four PCs explained approximately 48% of the variance in the data. Ordered contributions (%) of each behavioral variable are presented for the first four PCs: **(C)** PC1, **(D)** PC2, **(E)** PC3, and **(F)** PC4. For each variable, f and d denote frequency and duration, respectively. Significant contribution was set to 2.5%, as indicated by the red dashed line. Strongly contributing behaviors were considered as bring primarily responsible for changes in that PC: PC1 (orient, approach, follow, "face-to-face" display), PC2 (move away), PC3 (swing, circling), and PC4 (cut-off, wiggle).

Table S5. Loadings of each behavioral variable on to the first four PCs. Frequency (f) and duration (d) of behavior are indicated under type. Contributions greater than 2.5% from Fig. S1 are highlighted in bold. Behaviors that uniquely contribute to each PC are: PC1 (orient, approach, follow, “face-to-face” display), PC2 (move away), PC3 (swing, circling), and PC4 (cut-off, wiggle).

behavior	**type**	**PC1**	**PC2**	**PC3**	**PC4**
approach	f	**-0.31**	0.13	-0.04	0.12
	d	**-0.30**	0.08	-0.09	0.05
bite	f	-0.10	0.07	**0.32**	**-0.20**
chase	f	-0.14	**0.18**	**0.26**	**-0.19**
	d	-0.10	**0.16**	**0.29**	**-0.25**
follow	f	**-0.27**	0.08	0.04	0.04
	d	**-0.25**	0.08	0.04	0.05
gonopodial extension	f	0.04	**0.28**	0.13	**0.38**
	d	0.12	**0.24**	0.12	0.17
“cut-off”	f	-0.10	0.00	0.03	**-0.16**
	d	-0.10	0.01	0.04	**-0.17**
lunge	f	**-0.20**	0.11	**0.30**	-0.15
	d	**-0.18**	0.10	**0.26**	-0.13
orient	f	**-0.31**	0.12	-0.04	0.13
	d	**-0.19**	0.04	-0.13	0.04
skipping	f	-0.07	0.01	-0.10	0.12
	d	-0.06	0.00	-0.10	0.09
gonopodial swing or thrust	f	-0.13	-0.00	**0.17**	0.10
circling	f	-0.09	-0.07	**0.25**	0.06
	d	-0.09	-0.08	**0.24**	0.07
wiggle	f	0.05	0.01	-0.01	**-0.34**
	d	0.06	0.01	-0.01	**-0.33**
“face-to-face” display	f	**-0.16**	-0.14	-0.02	-0.02
	d	-0.13	-0.13	-0.06	-0.04
frontal display	f	**-0.26**	**-0.24**	-0.10	0.02
	d	**-0.22**	**-0.23**	-0.14	-0.07
lateral display	f	**-0.29**	**-0.19**	-0.06	0.04
	d	**-0.25**	**-0.20**	-0.09	-0.03
escape	f	0.09	**-0.26**	**0.23**	0.13
	d	0.09	**-0.25**	**0.23**	0.13
hiding	f	0.11	**-0.21**	0.20	-0.01
	d	0.11	**-0.21**	**0.14**	-0.06
move away	f	-0.07	**-0.30**	0.09	0.16
	d	-0.04	**-0.30**	0.13	**0.17**
bitten	f	0.03	**-0.23**	**0.27**	0.09
non-social	f	-0.03	**0.20**	0.06	**0.39**
	d	-0.02	-0.11	**-0.22**	**-0.18**

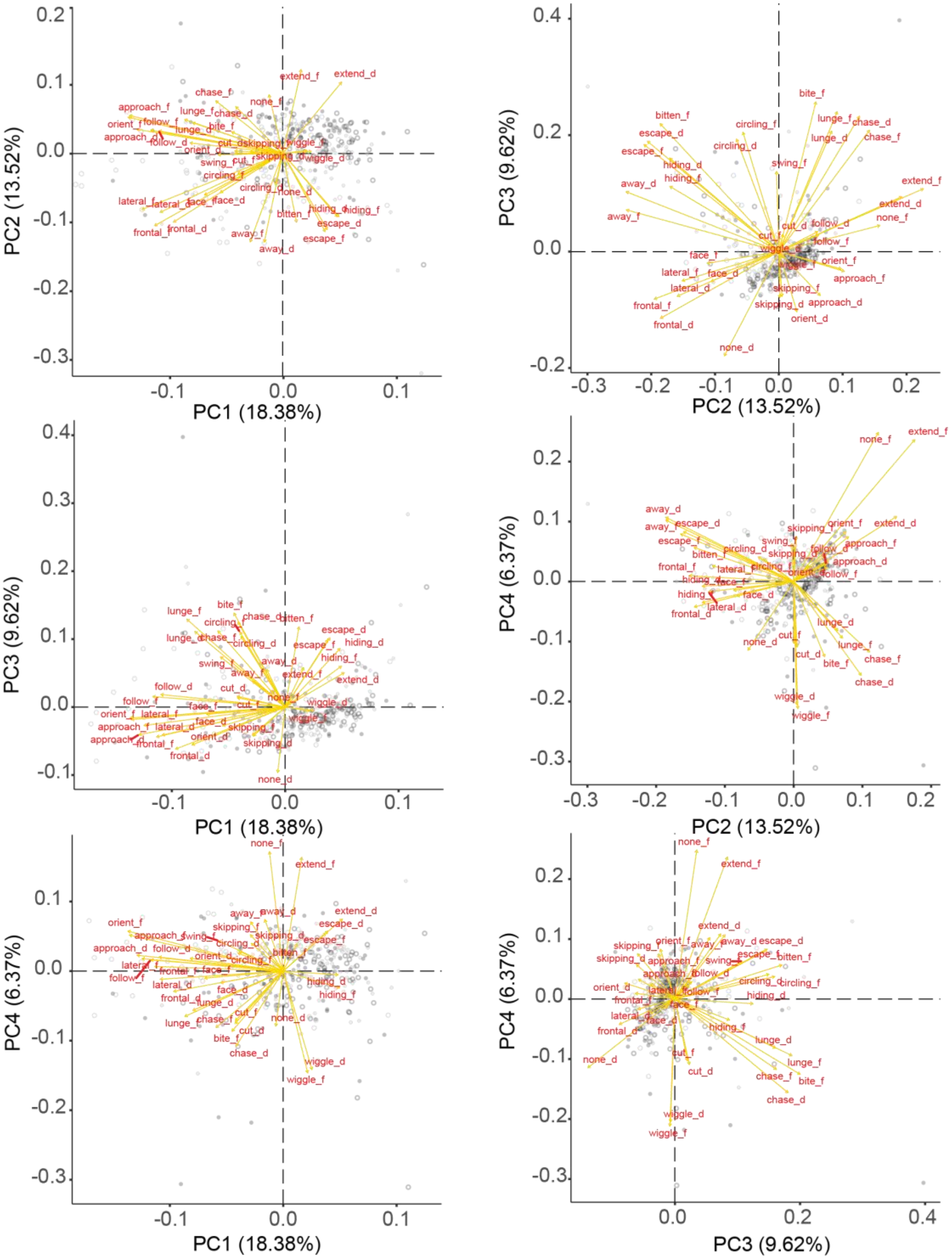

Figure S2. Bivariate plots for the first four PCs. Yellow arrows represent loadings. Dashed black lines show the 0 point for both PCs. Individual males are represented as points on the plot, where mottled are black and silver are gray. Assertive and submissive behaviors did not group together for most PC combinations. Certain behaviors dominate each PC: PC1 (orient, approach, follow, "face-to-face" display), PC2 (move away), PC3 (swing, circling), and PC4 (cut-off, wiggle).

Table S6. Bayesian mixed effects model for PC1 using a skewed normal distribution. Estimates, errors, and 95% credible intervals (CI) are presented for the random effects of focal and opponent male identity and all fixed effects. Male color morph is defined in parentheses: silver (s) or mottled (m). Social conflict outcome is noted as either dominant (dom) or subordinate (sub).

Formula: PC1 ~ relative size difference * focal color * opponent color * outcome
+ (1 | focal ID) + (1 | opponent ID)

	estimate	**error**	**CI**
Bayes R^2	0.28	0.04	/
Random			
focal ID: sd (intercept)	0.73	0.19	0.40 – 1.15
opponent ID: sd (intercept)	0.31	0.18	0.02 – 0.69
Fixed			
intercept	-1.55	0.42	-2.38 – -0.73
relative size difference	-0.09	1.16	-2.36 – 2.19
focal color (s)	0.68	0.51	-0.32 – 1.71
opponent color (s)	0.96	0.67	-0.37 – 2.25
outcome (dominant)	1.07	0.50	0.10 – 2.04
relative size difference * focal color (s)	0.48	1.41	-2.28 – 3.24
relative size difference * opponent color (s)	-0.80	1.58	-3.88 – 2.31
focal color (s) * opponent color (s)	0.64	0.73	-0.79 – 2.08
relative size difference * outcome (dom)	-0.01	1.54	-3.03 – 2.97
focal color (s) * outcome (dom)	0.39	0.79	-1.18 – 1.91
opponent color (s) * outcome (dom)	0.03	0.77	-1.47 – 1.54
relative size difference * focal color (s) * opponent color (s)	-0.55	1.81	-4.09 – 3.01
relative size difference * focal color (s) * outcome (dom)	-0.07	2.13	-4.24 – 4.13
relative size difference * opponent color (s) * outcome (dom)	-1.19	1.86	-4.82 – 2.47
focal color (s) * opponent color (s) * outcome (dom)	0.06	1.03	-1.95 – 2.10
relative size difference * focal color (s) * opponent color (s) * outcome (dom)	1.02	2.30	-3.51 – 5.56

Table S7. Contrasts for PC1 of dominant and subordinate male mosquitofish morphs across dyads. Contrasts are based on pairwise comparisons of the median posterior probability estimates and 0.95 credible interval of highest posterior probability density (HPD) with multivariate *t*-distribution adjustment. Contrasts are estimated using a relative size difference of 0 (i.e., size-matched). Dyads are displayed as dominant male > subordinate male. Male color morphs are indicated as silver (s) or mottled (m), and social status as dominant (dom) and subordinate (sub). Focal males whose behavior is being contrasted are in italics. Important contrasts (i.e., HPD does not cross 0) are also in bold. A higher PC1 means that the male uses more assertive behaviors (orient, approach, follow, “face-to-face” display). Dominant males of both morphs are more assertive towards silver males than mottled males. Dominant silver males are more assertive in within-morph interactions than any other dyad-type. Generally, dominant males are more assertive than subordinate males, except dominant silver males towards subordinate mottled males. Subordinate silver males are less assertive with mottled males than silver males, and less assertive than subordinate mottled males with mottled males.

	contrast	**estimate**	**lower HPD**	**upper HPD**
dominant				
	(*M dom* > M sub) - (*S dom* > M sub)	-1.08	-2.52	0.38
	(*M dom* > M sub) - (*M dom* > S sub)	**-0.99**	**-1.86**	**-0.15**
	(*M dom* > M sub) - (*S dom* > S sub)	**-2.75**	**-3.98**	**-1.60**
	(*S dom* > M sub) - (*M dom* > S sub)	0.09	-1.39	1.53
	(*S dom* > M sub) - (*S dom* > S sub)	**-1.68**	**-3.07**	**-0.35**
	(*M dom* > S sub) - (*S dom* > S sub)	**-1.76**	**-2.76**	**-0.73**
subordinate				
	(M dom > *M sub*) - (M dom > *S sub*)	-0.68	-1.73	0.29
	(M dom > *M sub*) - (S dom > *M sub*)	-0.96	-2.29	0.33
	(M dom > *M sub*) - (S dom > *S sub*)	**-2.28**	**-3.48**	**-1.07**
	(M dom > *S sub*) - (S dom > *M sub*)	-0.28	-1.74	1.21
	(M dom > *S sub*) - (S dom > *S sub*)	**-1.60**	**-2.47**	**-0.71**
	(S dom > *M sub*) - (S dom > *S sub*)	-1.31	-2.80	0.15
social status				
	(*M dom* > M sub) - (M dom > *M sub*)	**-1.07**	**-2.03**	**-0.09**
	(M dom > *S sub*) - (*S dom* > M sub)	**-1.47**	**-2.77**	**-0.15**
	(M dom > *S sub*) - (*M dom* > S sub)	**-1.38**	**-2.32**	**-0.44**
	(S dom > *M sub*) - (*S dom* > M sub)	-1.19	-3.03	0.57
	(S dom > *M sub*) - (*M dom* > S sub)	-1.10	-2.37	0.20
	(*S dom* > S sub) - (S dom > *S sub*)	**-1.55**	**-2.51**	**-0.53**

Table S8. Bayesian mixed effects model for PC2 using a skewed normal distribution. Estimates, errors, and 95% credible intervals (CI) are presented for the random effects of focal and opponent male identity and all fixed effects. Male color is defined in parentheses: silver (s) or mottled (m). Social conflict outcome is noted as either dominant (dom) or subordinate (sub).

Formula: PC2 ~ relative size difference * focal color * opponent color * outcome + (1 | focal ID) + (1 | opponent ID)

	estimate	**error**	**CI**
Bayes R^2	0.29	0.03	/
Random			
focal ID: sd (intercept)	0.53	0.16	0.24 – 0.86
opponent ID: sd (intercept)	0.36	0.16	0.04 – 0.69
Fixed			
intercept	-0.74	0.34	-1.41 – -0.06
relative size difference	0.05	0.92	-1.74 – 1.85
focal color (s)	-0.16	0.40	-0.94 – 0.61
opponent color (s)	-0.43	0.55	-1.49 – 0.66
outcome (dominant)	1.53	0.41	0.72 – 2.33
relative size difference * focal color (s)	-0.04	1.12	-2.24 – 2.19
relative size difference * opponent color (s)	-1.00	1.29	-3.57 – 1.52
focal color (s) * opponent color (s)	-0.28	0.59	-1.44 – 0.86
relative size difference * outcome (dom)	0.82	1.30	-1.74 – 3.37
focal color (s) * outcome (dom)	0.30	0.66	-0.96 – 1.61
opponent color (s) * outcome (dom)	0.65	0.62	-0.59 – 1.86
relative size difference * focal color (s) * opponent color (s)	0.24	1.50	-2.72 – 3.20
relative size difference * focal color (s) * outcome (dom)	0.79	1.82	-2.79 – 4.36
relative size difference * opponent color (s) * outcome (dom)	0.06	1.57	-3.01 – 3.14
focal color (s) * opponent color (s) * outcome (dom)	-0.38	0.85	-2.07 – 1.29
relative size difference * focal color (s) * opponent color (s) * outcome (dom)	0.00	1.94	-3.83 – 3.81

Table S9. Contrasts for PC2 of dominant and subordinate male mosquitofish morphs across dyads. Contrasts are based on pairwise comparisons of the median posterior probability estimates and 0.95 credible interval of highest posterior probability density (HPD) with multivariate *t*-distribution adjustment. Contrasts are estimated using a relative size difference of 0 (i.e., size-matched). Dyads are displayed as dominant male > subordinate male. Male color morphs are indicated as silver (s) or mottled (m), and social status as dominant (dom) and subordinate (sub). Focal males whose behavior is being contrasted are in italics. Important contrasts (i.e., HPD does not cross 0) are also in bold. All subordinate males move away more (lower PC2) from dominant males.

	contrast	**estimate**	**lower HPD**	**upper HPD**
dominant				
	(*M dom* > M sub) - (*S dom* > M sub)	-0.13	-1.33	1.06
	(*M dom* > M sub) - (*M dom* > S sub)	-0.22	-0.92	0.53
	(*M dom* > M sub) - (*S dom* > S sub)	0.30	-0.67	1.25
	(*S dom* > M sub) - (*M dom* > S sub)	-0.09	-1.32	1.14
	(*S dom* > M sub) - (*S dom* > S sub)	0.43	-0.73	1.62
	(*M dom* > S sub) - (*S dom* > S sub)	0.52	-0.28	1.31
subordinate				
	(M dom > *M sub*) - (M dom > *S sub*)	0.15	-0.62	0.93
	(M dom > *M sub*) - (S dom > *M sub*)	0.43	-0.64	1.51
	(M dom > *M sub*) - (S dom > *S sub*)	0.86	-0.07	1.85
	(M dom > *S sub*) - (S dom > *M sub*)	0.28	-0.89	1.46
	(M dom > *S sub*) - (S dom > *S sub*)	0.70	-0.02	1.44
	(S dom > *M sub*) - (S dom > *S sub*)	0.43	-0.68	1.62
social status				
	(*M dom* > M sub) - (M dom > *M sub*)	**-1.53**	**-2.32**	**-0.71**
	(M dom > *S sub*) - (*S dom* > M sub)	**-1.82**	**-2.96**	**-0.74**
	(M dom > *S sub*) - (*M dom* > S sub)	**-1.91**	**-2.67**	**-1.13**
	(S dom > *M sub*) - (*S dom* > M sub)	**-2.10**	**-3.57**	**-0.59**
	(S dom > *M sub*) - (*M dom* > S sub)	**-2.18**	**-3.23**	**-1.12**
	(*S dom* > S sub) - (S dom > *S sub*)	**-2.10**	**-2.89**	**-1.29**

Table S10. Bayesian mixed effects model for PC3 using a skewed normal distribution. Estimates, errors, and 95% credible intervals (CI) are presented for the random effect of focal and opponent male identity and all fixed effects. Male color is defined in parentheses: silver (s) or mottled (m). Social conflict outcome is noted as either dominant (dom) or subordinate (sub).

Formula: PC3 ~ relative size difference * focal color * opponent color * outcome + (1 | focal ID) + (1 | opponent ID)

	estimate	**error**	**CI**
Bayes R^2	0.08	0.02	/
Random			
focal ID: sd (intercept)	0.33	0.13	0.09 – 0.60
opponent ID: sd (intercept)	0.25	0.14	0.02 – 0.54
Fixed			
intercept	0.10	0.26	-0.43 – 0.59
relative size difference	0.20	0.72	-1.20 – 1.62
focal color (s)	0.09	0.30	-0.50 – 0.67
opponent color (s)	0.56	0.41	-0.26 – 1.35
outcome (dominant)	-0.20	0.30	-0.79 – 0.41
relative size difference * focal color (s)	0.39	0.87	-1.36 – 2.08
relative size difference * opponent color (s)	0.04	1.07	-2.01 – 2.17
focal color (s) * opponent color (s)	-0.48	0.45	-1.35 – 0.40
relative size difference * outcome (dom)	0.73	1.00	-1.22 – 2.69
focal color (s) * outcome (dom)	0.92	0.48	-0.06 – 1.85
opponent color (s) * outcome (dom)	-0.39	0.47	-1.31 – 0.53
relative size difference * focal color (s) * opponent color (s)	0.65	1.22	-1.72 – 3.04
relative size difference * focal color (s) * outcome (dom)	-0.97	1.44	-3.82 – 1.86
relative size difference * opponent color (s) * outcome (dom)	-0.43	1.23	-2.89 – 1.96
focal color (s) * opponent color (s) * outcome (dom)	-0.62	0.66	-1.90 – 0.69
relative size difference * focal color (s) * opponent color (s) * outcome (dom)	-0.56	1.57	-3.62 – 2.55

Table S11. Contrasts for PC3 of dominant and subordinate male mosquitofish morphs across dyads. Contrasts are based on pairwise comparisons of the median posterior probability estimates and 0.95 credible interval of highest posterior probability density (HPD) with multivariate *t*-distribution adjustment. Contrasts are estimated using a relative size difference of 0 (i.e., size-matched). Dyads are displayed as dominant male > subordinate male. Male color morphs are indicated as silver (s) or mottled (m), and social status as dominant (dom) and subordinate (sub). Focal males whose behavior is being contrasted are in italics. Important contrasts (i.e., HPD does not cross 0) are also in bold. Dominant silver males with subordinate silver males have a higher PC3 (more swings and circling) than with subordinate mottled males, and a higher PC3 than dominant mottled males with subordinate mottled males.

	contrast	**estimate**	**lower HPD**	**upper HPD**
dominant				
	(*M dom* > M sub) - (*S dom* > M sub)	**-1.02**	**-1.86**	**-0.16**
	(*M dom* > M sub) - (*M dom* > S sub)	-0.16	-0.74	0.39
	(*M dom* > M sub) - (*S dom* > S sub)	-0.06	-0.78	0.69
	(*S dom* > M sub) - (*M dom* > S sub)	0.86	-0.05	1.71
	(*S dom* > M sub) - (*S dom* > S sub)	**0.96**	**0.09**	**1.83**
	(*M dom* > S sub) - (*S dom* > S sub)	0.09	-0.54	0.73
subordinate				
	(M dom > *M sub*) - (M dom > *S sub*)	-0.09	-0.67	0.50
	(M dom > *M sub*) - (S dom > *M sub*)	-0.56	-1.35	0.26
	(M dom > *M sub*) - (S dom > *S sub*)	-0.17	-0.88	0.57
	(M dom > *S sub*) - (S dom > *M sub*)	-0.47	-1.33	0.39
	(M dom > *S sub*) - (S dom > *S sub*)	-0.08	-0.66	0.52
	(S dom > *M sub*) - (S dom > *S sub*)	0.39	-0.44	1.25
social status				
	(*M dom* > M sub) - (M dom > *M sub*)	0.20	-0.40	0.79
	(M dom > *S sub*) - (*S dom* > M sub)	-0.73	-1.52	0.08
	(M dom > *S sub*) - (*M dom* > S sub)	0.13	-0.46	0.68
	(S dom > *M sub*) - (*S dom* > M sub)	-0.26	-1.32	0.83
	(S dom > *M sub*) - (*M dom* > S sub)	0.60	-0.18	1.34
	(*S dom* > S sub) - (S dom > *S sub*)	0.30	-0.43	0.98

Table S12. Bayesian mixed effects model for PC4 using a skewed normal distribution. Estimates, errors, and 95% credible intervals (CI) are presented for the random effect of focal and opponent male identity and all fixed effects. Male color is defined in parentheses: silver (s) or mottled (m). Social conflict outcome is noted as either dominant (dom) or subordinate (sub).

Formula: PC4 ~ relative size difference * focal color * opponent color * outcome + (1 | focal ID) + (1 | opponent ID)

	estimate	**error**	**CI**
Bayes R^2	0.18	0.03	/
Random			
focal ID: sd (intercept)	0.65	0.15	0.41 – 1.00
opponent ID: sd (intercept)	0.12	0.08	0.00 – 0.31
Fixed			
intercept	0.52	0.28	-0.03 – 1.08
relative size difference	-0.21	0.66	-1.50 – 1.08
focal color (s)	-0.58	0.36	-1.29 – 0.12
opponent color (s)	-0.50	0.38	-1.25 – 0.22
outcome (dominant)	0.08	0.28	-0.46 – 0.62
relative size difference * focal color (s)	-0.40	0.81	-1.97 – 1.18
relative size difference * opponent color (s)	0.30	0.86	-1.35 – 2.00
focal color (s) * opponent color (s)	0.02	0.42	-0.79 – 0.84
relative size difference * outcome (dom)	0.09	0.83	-1.54 – 1.71
focal color (s) * outcome (dom)	0.15	0.45	-0.75 – 1.03
opponent color (s) * outcome (dom)	0.13	0.43	-0.71 – 0.99
relative size difference * focal color (s) * opponent color (s)	-0.53	0.99	-2.47 – 1.42
relative size difference * focal color (s) * outcome (dom)	-0.64	1.17	-2.94 – 1.66
relative size difference * opponent color (s) * outcome (dom)	0.37	0.99	-1.58 – 2.30
focal color (s) * opponent color (s) * outcome (dom)	0.30	0.59	-0.85 – 1.46
relative size difference * focal color (s) * opponent color (s) * outcome (dom)	-0.16	1.23	-2.58 – 2.24

Table S13. Contrasts for PC4 of dominant and subordinate male mosquitofish morphs across dyads. Contrasts are based on pairwise comparisons of the median posterior probability estimates and 0.95 credible interval of highest posterior probability density (HPD) with multivariate *t*-distribution adjustment. Contrasts are estimated using a relative size difference of 0 (i.e., size-matched). Dyads are displayed as dominant male > subordinate male. Male color morphs are indicated as silver (s) or mottled (m), and social status as dominant (dom) and subordinate (sub). Focal males whose behavior is being contrasted are in italics. Important contrasts (i.e., HPD does not cross 0) are also in bold. In interactions with dominant mottled males, subordinate silver males have a lower PC4 (fewer cut-offs and wiggles) than subordinate mottled males.

	contrast	**estimate**	**lower HPD**	**upper HPD**
dominant				
	(*M dom* > M sub) - (*S dom* > M sub)	0.43	-0.48	1.37
	(*M dom* > M sub) - (*M dom* > S sub)	0.37	-0.09	0.82
	(*M dom* > M sub) - (*S dom* > S sub)	0.49	-0.28	1.28
	(*S dom* > M sub) - (*M dom* > S sub)	-0.05	-0.99	0.89
	(*S dom* > M sub) - (*S dom* > S sub)	0.06	-0.76	0.81
	(*M dom* > S sub) - (*S dom* > S sub)	0.11	-0.59	0.84
subordinate				
	(M dom > *M sub*) - (M dom > *S sub*)	0.58	-0.11	1.30
	(M dom > *M sub*) - (S dom > *M sub*)	0.50	-0.23	1.24
	(M dom > *M sub*) - (S dom > *S sub*)	**1.07**	**0.29**	**1.86**
	(M dom > *S sub*) - (S dom > *M sub*)	-0.09	-1.01	0.83
	(M dom > *S sub*) - (S dom > *S sub*)	0.48	-0.01	0.97
	(S dom > *M sub*) - (S dom > *S sub*)	0.57	-0.35	1.49
social status				
	(*M dom* > M sub) - (M dom > *M sub*)	-0.08	-0.62	0.46
	(M dom > *S sub*) - (*S dom* > M sub)	-0.24	-0.98	0.57
	(M dom > *S sub*) - (*M dom* > S sub)	-0.29	-0.98	0.35
	(S dom > *M sub*) - (*S dom* > M sub)	-0.15	-1.22	0.99
	(S dom > *M sub*) - (*M dom* > S sub)	-0.20	-0.93	0.53
	(*S dom* > S sub) - (S dom > *S sub*)	**-0.66**	**-1.25**	**-0.10**

Table S14. Behavioral transitions that contribute significantly to the social behavior sequence used by dominant male mosquitofish morphs across dyads. Counts are pooled across trials. Frequencies are calculated based on all possible transitions. Z-scores are calculated based median absolute deviation for transitions with frequencies > 0.

focal	**stimulus**	**transition**	**count**	**frequency**	**z**
silver	*silver*	approach → frontal display	180	0.013	26.69
		approach → gonopodial extension	224	0.016	33.36
		approach → lateral display	196	0.014	29.12
		approach → orient	396	0.029	59.45
		gonopodial extension → orient	654	0.048	98.57
		lateral display → gonopodial extension	124	0.009	18.20
		orient → approach	1537	0.113	232.48
		orient → gonopodial extension	164	0.012	24.26
silver	*mottled*	approach → frontal display	29	0.007	12.28
		approach → gonopodial extension	140	0.033	62.78

		approach → lateral display	51	0.012	22.29
		approach → orient	78	0.018	34.58
		gonopodial extension → orient	347	0.082	156.96
		lateral display → gonopodial extension	59	0.014	25.93
		orient → approach	449	0.105	203.36
		orient → gonopodial extension	59	0.014	25.93
mottled	*mottled*	approach → frontal display	71	0.007	10.16
		approach → gonopodial extension	222	0.021	33.06
		approach → lateral display	110	0.01	16.07
		approach → orient	296	0.027	44.28
		gonopodial extension → orient	559	0.052	84.16
		lateral display → gonopodial extension	93	0.009	13.50
		orient → approach	1061	0.099	160.29
		orient → gonopodial extension	166	0.015	24.57
mottled	*silver*	approach → frontal display	230	0.010	34.27
		approach → gonopodial extension	504	0.022	75.82
		approach → lateral display	296	0.013	44.28
		approach → orient	724	0.031	109.19
		gonopodial extension → orient	1239	0.054	187.29
		lateral display → gonopodial extension	216	0.009	32.15
		orient → approach	2500	0.108	378.51
		orient → gonopodial extension	384	0.017	57.63

Table S15. Comparison of social behavior transitions use by dominant male mosquitofish morphs towards same- or different-color males in dyadic encounters. Results of the Kruskal-Wallis ANOVAs are presented. Significant differences are highlighted in bold for P-values below the Bonferroni-adjusted alpha (0.006). Dominant males in at least one dyad differed significantly in their use of the transition *approach → frontal display*.

transition	chi-squared	df	P-value
approach → frontal display	**14.14**	**3**	**0.003**
approach → gonopodial extension	7.21	3	0.066
approach → lateral display	5.78	3	0.123
approach → orient	8.16	3	0.043
gonopodial extension → orient	6.84	3	0.077
lateral display → gonopodial extension	1.48	3	0.687
orient → approach	4.27	3	0.234
orient → gonopodial extension	2.81	3	0.421

Table S16. Contrasts of significantly different behavioral transitions for dominant male mosquitofish morphs towards same- or different-color males in dyadic encounters. Transition use is compared for dominant male morphs across dyads using Wilcoxon-ranked pairwise *post-hoc* tests. Dyads are indicated as focal morph > opponent morph. Males are either mottled (m) or silver (s). Significant differences are highlighted in bold for Bonferroni-adjusted P-values. In within-morph interactions, dominant silver males use the behavioral transition *approach → frontal display* significantly more than dominant mottled males.

transition	contrast 1	contrast 2	P-value
approach → frontal display			
	m > s	m > m	0.24
	s > m	m > m	1.00
	s > s	**m > m**	**0.002**
	s > m	m > s	1.00
	s > s	m > s	0.22
	s > s	s > m	0.19

Table S17. Behavioral transitions that contribute significantly to the social behavior sequence used by subordinate male mosquitofish morphs across dyads. Counts are pooled across trials. Frequencies are calculated based on all possible transitions. Z-scores are calculated based median absolute deviation for transitions with frequencies > 0.

focal	**stimulus**	**transition**	**count**	**frequency**	**z**
silver	*silver*	approach → frontal display	125	0.010	18.35
		approach → gonopodial extension	144	0.012	21.23
		approach → lateral display	171	0.014	25.33
		approach → orient	307	0.025	45.95
		frontal display → lateral display	141	0.012	20.78
		gonopodial extension → orient	436	0.036	65.51
		orient → approach	1095	0.091	165.45
		orient → gonopodial extension	111	0.009	16.23
silver	*mottled*	approach → frontal display	157	0.008	35.03
		approach → gonopodial extension	207	0.011	46.40
		approach → lateral display	180	0.009	40.26
		approach → orient	337	0.018	75.98
		frontal display → lateral display	148	0.008	32.98
		gonopodial extension → orient	642	0.034	145.35
		orient → approach	1450	0.076	329.15
		orient → gonopodial extension	209	0.011	46.86
mottled	*mottled*	approach → frontal display	56	0.006	12.06
		approach → gonopodial extension	87	0.009	19.11
		approach → lateral display	95	0.010	20.93
		approach → orient	214	0.023	48.00
		frontal display → lateral display	69	0.008	15.01
		gonopodial extension → orient	268	0.029	60.28
		orient → approach	734	0.080	166.28
		orient → gonopodial extension	74	0.008	16.15
mottled	*silver*	approach → frontal display	26	0.008	5.23
		approach → gonopodial extension	53	0.017	11.37
		approach → lateral display	36	0.011	7.51
		approach → orient	80	0.025	17.52
		frontal display → lateral display	22	0.007	4.32
		gonopodial extension → orient	147	0.046	32.76
		orient → approach	303	0.095	68.24
		orient → gonopodial extension	46	0.014	9.78

Table S18. Comparison of significantly contributing social behavioral transitions for subordinate male mosquitofish morphs across dyads. Results of the Kruskal-Wallis ANOVAs are presented. No significant differences were highlighted because no P-value was below the Bonferroni-adjusted alpha (0.006). Subordinate morphs did not differ in behavioral sequence across dyads.

transition	**chi-squared**	**df**	**p-value**
approach → frontal display	6.29	3	0.10
approach → gonopodial extension	1.10	3	0.78
approach → lateral display	7.36	3	0.06
approach → orient	8.55	3	0.04
frontal display → lateral display	4.73	3	0.19
gonopodial extension → orient	2.29	3	0.52
orient → approach	3.20	3	0.36
orient → gonopodial extension	2.08	3	0.56

CHAPTER 5

Male Eastern Mosquitofish (*Gambusia holbrooki*) Exhibit Morph-Specific Neuromolecular Responses to Acute Social Interactions

5.1 Abstract

Across taxa, morphological and behavioral traits can correlate within individuals through a variety of molecular mechanisms. Male Eastern Mosquitofish (*Gambusia holbrooki*) exhibit a discrete color polymorphism in which males are either the common silver morph or the uncommon mottled morph, unpigmented with black blotches. Mottled males dominate silver males in social interactions across a range of size differences and social contexts. The underlying mechanisms for the correlation of social dominance and this melanin-based spotting pattern in *G. holbrooki* is unknown. Both pigmentation and behavior are complex phenotypes that involve multiple genes across different systems. I used a systems biology approach to explore potential molecular candidates that regulate the mottled morph. Neuromolecular response to morph-dependent social interaction was recorded using RNA-sequencing of whole brains for mottled males and silver males following staged social interactions with silver males, the common morph of male social partners in the wild. Following the creation of the first transcriptome for this species, I identified 563 differentially expressed transcripts between morphs. Male morphs showed clear and distinct differences in their neuromolecular responses. I identified five transcripts of interest that could be involved in social behavior according to their gene ontology functions: the activin A receptor type 2A (*acvr2aa*), calcium channel, voltage-dependent, gamma subunit 7a (*cacng7a*), disks large homolog 4-like (*dlg4*), GIPC PDZ domain containing family, member 1 (*gipc1*), and kirre like nephrin family adhesion

molecule 3b (*kirrel3b*). Expression of *cacng7a* and *gipc1* transcripts correlated with aggression frequency and duration, respectively, while *kirrel3b* correlated with submission duration. The transcripts *acvr2aa*, *cacng7a*, and *gipc1* also function in the regulation of melanin pigmentation, indicating their strong potential as mechanisms for the correlation between color pattern and social behavior in morphs of male *G. holbrooki*. Furthermore, the presence of the mottled color pattern in *G. holbrooki* was previously determined to be associated with the Y-linked gene *gipc1*. Overall, this study identified potential candidates for future study of the neuromolecular mechanisms underlying the phenotypic correlation of social behavior and pigmentation in male Eastern Mosquitofish.

5.2 Introduction

Across vertebrates, melanin-based color polymorphisms correlate with differences in other traits, such as behavior (rev: Ducrest *et al*., 2008; McKinnon and Pierotti, 2010; San-Jose and Roulin, 2018). In the family Poeciliidae, many species exhibit color polymorphisms; this family has thus been identified as an amenable model system for the study of phenotypic correlations (Zerulla and Stoddard, 2021). Within Poeciliids, Eastern Mosquitofish (*Gambusia holbrooki*) exhibit a heritable, sex-linked discrete color polymorphism (Regan, 1961; Angus, 1989; Horth, 2006; Kottler *et al*., 2020). Male mosquitofish express either the common, unspotted silver pattern or the uncommon mottled pattern, unpigmented with black or brown blotches over the whole body (Myers, 1925; Regan, 1961; Angus, 1989; Horth, 2004). At this time, no specific gene that regulates the mottled pattern has been definitively identified, but alleles of *GIPC1* (GIPC PDZ domain containing family member 1) were associated with this phenotype by a different method, restriction-site associated DNA (RAD-) and pool sequencing *(Kottler et* *al*., 2020). These color morphs also differ in the expression of social behavior (rev: Zerulla and Stoddard, 2021). Specifically, mottled males dominate silver males regardless of size difference or social context (*Chapters 3* and *4*). Since the mottled pattern is heritable, the covariation of social behavior and pigmentation in male mosquitofish morphs may have a genetic basis.

The specific molecular pathways that regulate the covarying expression of this melanin-based color pattern and social behavior in male mosquitofish are unknown. Previous studies in other species used candidate gene approaches that often focused on the melanocortin system (rev: Ducrest *et al*., 2008; San-Jose and Roulin, 2017, 2018). Melanocortins are pituitary peptide hormones that regulate melanin synthesis and distribution within a cell, as well as pleiotropically affect social behavior (rev: Ducrest *et al*., 2008; San-Jose and Roulin, 2018); however, melanocortin differences often only explained the color polymorphism and not behavioral differences (rev: San-Jose and Roulin, 2017). Melanin-based pigmentation and social behavior are both complex phenotypes that involve multiple genes across different systems, including neuroendocrine systems (e.g., monoamines, amino acid hormones, sex steroids, and other peptide hormones), developmental systems (e.g., neural crest differentiation), and possibly non protein-coding mechanisms, such as epigenetic regulation of transcription (rev: Ducrest *et al*., 2008; Wilkins *et al*., 2014; San-Jose and Roulin, 2017, 2020; Zerulla and Stoddard, 2021). Because the mechanisms regulating male mosquitofish color patterns and social behavior are likely complex, I focused on a systems biology approach using transcriptomics.

Transcriptomics has been used to investigate neuromolecular differences for color morphs that differ in social behavior in other species. Male black-faced blennies (*Tripterygion delaisi*) are either “sneaker” males or territorial males that undergo a color change (Schunter *et al*., 2014). Some differentially expressed transcripts between these morphs were identified as important to both color and behavior using RNA-Sequencing. Similarly, giant freshwater prawns (*Macrobarachium rosenbergii*) exhibit three morphs that are hypothesized to be the result of differences in social dominance: small and transparent-clawed males, orange-clawed males, and dominant blue-clawed males (Aziz *et al*., 2017). Differentially expressed transcripts that could directly or indirectly affect both pigmentation and behavior in these prawns were identified through RNA-Sequencing. I used whole-brain RNA-sequencing to explore neuromolecular differences between mottled and silver males following an acute social interaction with a silver male, the most common morph for male social interactions in the wild.

5.3 Methods

5.3.1 Experimental Fish

Eastern Mosquitofish (*Gambusia holbrooki*) were collected from Student Housing Pond (25°45'24.6"N 80°22'14.8"W) on the MMC Campus at Florida International University (FIU) in Miami, Florida, U.S.A. Fish were housed at 22°C in mixed-sex and mixed-color groups in 38-L aquaria with limestone pea gravel and PVC shelters under natural daylight hours (12 light:12 dark). Each fish was fed 0.01 grams of TetraColor Plus Tropical Fish Flakes daily.

Before behavioral testing, 16 experimental fish (four mottled males and 12 silver males) were isolated in separate 19-L aquaria for at least 24 hours. Standard length (tip of snout to end of caudal peduncle) was measured using a digital caliper and all fish were size-matched to avoid confounding effects of size on social behavior (22.5±0.5 mm). To measure the amount of melanin-based pigmentation, photographs of each fish's lateral sides were traced in Adobe *Fresco* (2022) to create black-and-white images of the pigmentation patterns. These images were analyzed for proportion of pigmentation on total body area using *ImageJ* software (v.1.52k; Schneider *et al.*, 2012), with the final value as an average of the left and right side of the fish (0.64±0.1).

Because silver males have no distinct morphological features that can be used to distinguish between individuals on video, fish were freeze-branded for identification. Males were placed in a 1.9-L plastic holding aquarium, then lifted for a few seconds in a net to brand a small spot on its side using a copper wire chilled in liquid nitrogen. Fish were returned to the holding aquarium and immersed in water containing Stress Coat (API). Males were allowed to recover for 30 min afterwards, and all fish survived the freeze-branding and swam normally within 5 min. This freeze-branding method did not work on the skin of mottled males, so their natural individually distinctive color patterns were used to identify individuals. Mottled males were still handled on the same day as silver males. All animal care and protocols were approved under FIU's IACUC-18-039, IACUC-17-012, IACUC-17-012-CR01, and IACUC-17-012-AM01.

5.3.2 Social Behavior Test

To test for genes in the brain that may underlie the correlation between social behavior and color pattern in *G. holbrooki*, males of each morph were paired with a silver male in dyadic encounters. Silver males were chosen as the social partner because they are the most common male morph with which both morphs interact in the wild. Fish acclimated in 1.9-L plastic holding aquaria for 5 min prior to testing. Experimental fish were moved into a behavioral arena that consisted of a 19-L aquarium (40 cm long x 25 cm high x 21 cm deep) with 1-inch of limestone pea gravel on the bottom. Two fluorescent lights were positioned on either side of the aquarium to illuminate it. To reduce reflection on the sides of the aquarium that caused males to see and preferentially interact with their own reflection, white translucent plastic binder dividers were placed inside against the glass. A white foam board was placed on the back of the aquarium to provide contrast for observing the fish. A GoPro Hero4 camera (1080p, 60 fps) video recorded the behavior for 30 min. In the fish *Betta splendens*, consistent neuromolecular states for fish engaged in agonistic encounters occurred after 30 minutes (Vu *et al*., 2021), so this duration was chosen for acute social conflict in this experiment. After recording began, the area was covered with a black cloth to avoid disturbing the fishes' behavior. Water was replaced between behavioral tests to maintain the temperature at 22°C and to prevent the possible effect of odorants or other released chemicals on the next test.

Social behavior was scored in BORIS (v.7.10.2; Friard and Gamba, 2016) based on the standardized *G. holbrooki* ethogram (*Chapter 3*) for the entire 30 min video. Both the frequency and duration of individual behaviors were recorded for state events, while only frequency was recorded for point events. Behavior frequency and duration (s) was also summed for all behaviors in each category: aggression, submission, and non-social. A dominance index was calculated for each male: [duration of focal male aggression] / [duration of focal male aggression + duration of focal male submission]. If the focal male had a higher dominance index than his opponent, then he was considered dominant. If the males had equal dominance indices, the male with a higher duration of aggression was considered dominant.

5.3.3 Sample Preparation and Sequencing

Focal males were euthanized in buffered MS-222 solution immediately after the behavioral test. Whole brains were dissected following the protocol by Gupta and Mullins (2010). Sections were stored in RNAlater and frozen at -20°C for processing. RNA was extracted using the Monarch Total RNA Miniprep Kit (New England Biolabs). An additional wash with RNA Wash Buffer was added to their protocol to remove salts from the extracted RNA. Following extraction, samples were tested on both Nanodrop and Qubit to check the RNA concentration (ng/uL) and quality. Since genomic DNA was detected in our samples following RNA extraction, I treated all samples with TurboDNase to break down the genomic DNA to prevent its interference in downstream sequencing. Finally, RNA samples were tested on an Agilent BioAnalyzer to confirm quality, of which the highest quality three samples for each morph were sent to Novogene for sequencing with the Illumina HiSeq 4000 (stranded paired-end 150 bp). An overview of sample information, quality, and sequencing statistics is available in the Appendix (Table S1).

5.3.4 Bioinformatic Analysis

After confirming the quality of all sample libraries using FastQC v.0.11.9 (Andrew, 2010), libraries were trimmed with Trimmomatic v.0.39 (Bolger *et al*., 2014). Libraries were aligned against the *Gambusia holbrooki* genome (Kottler *et al*., 2020) with HiSAT2 v.2.2.1 (Kim *et al*., 2019) to remove contaminating and poor quality sequences from our libraries. These reduced libraries were used to create a genome-guided transcriptome with

Trinity v.2.13.2 (Haas *et al*., 2013). This transcriptome assembly had 376,135 sequences and may include redundant transcripts. Sequences were clustered using a 95% similarity threshold using CD-HIT-EST v.4.8.1 (Li and Godzik, 2006; Fu *et al*., 2012). A quality check for this assembly was performed with gVolante v.2.0.0 and BUSCO v.5 (Simão *et al*., 2015; Nishimura *et al*., 2017). This genome-guided assembly determined that 92.7% of core genes queried were completely present and an additional 5.1% were partially present. The total number of sequences for the genome-guided transcriptome assembly is 187,914 with an N50 sequence length of 2585 nt and a GC-content of 45.76%, indicating a high-quality assembly.

Sample libraries were aligned to the transcriptome and quantified using Salmon v.1.5.2 (Patro *et al*., 2017). Differential gene expression analysis and visualization was conducted in R v.4.2.3 (R Core Team, 2021). Count tables were imported using tximport v.1.26.1 (Soneson *et al*., 2015) for analysis with DESeq2 v.1.38.3 (Love *et al*., 2014). A Principal Components Analysis (PCA) plot for all genes revealed that social status should be included to cluster samples by color morph (Fig. S1). The negative binomial model was specified to include social status as an effect on differences in gene expression between male color morphs. Differentially expressed genes were identified using a false discovery rate (FDR) <0.05. Data visualization was performed with ggplot2 from tidyverse v.2.0.0 (Wickham *et al*., 2019), pheatmap v.1.0.12 (Kolde, 2019), and Adobe *Illustrator* (2023). These analyses were based on a tutorial from the Harvard Chan Bioinformatics Core (Mistry *et al*., 2021).

Because the *G. holbrooki* genome annotation's current file form was not usable by the necessary bioinformatic software, I have not yet completed a full annotation of the transcriptome. For efficiency, only the top 50 differentially expressed genes were considered for this chapter. Transcript identity was confirmed using BLAST software against the NCBI nucleotide database (Altschul *et al.*, 1990). Identity was based on the closest related species with >90% similarity in transcript sequence. Standard practice for the accurate annotation of gene function is to use gene ontology terms instead of individual literature citations (Hill *et al.*, 2008). Gene ontology terms are based on curated annotations from the literature and computational inferences (Ashburner *et al.*, 2000; Hill et al., 2008; The Gene Ontology Consortium, 2021). Transcript functions were identified through their gene ontology terms listed in the 2023_01 release of the UniprotKB database (The UniProt Consortium, 2023). Where possible, closely related fish species and the Zebrafish (*Danio rerio*) were used to determine function. If the annotation was not available for fish, mouse (*Mus musculus*) or human (*Homeo sapiens*) were used. Finally, five genes associated with synaptic transmission that potentially affect social behavior according to the literature were tested in a Pearson correlation matrix against behavior (frequency and duration of aggression and submission, dominance index, and relative dominance).

5.4 Results

The sampled male *G. holbrooki* color morphs have 563 differentially expressed transcripts, of which 308 are up-regulated and 255 are down-regulated in mottled males relative to silver males (Fig. 1). The expression profiles for these differentially expressed transcripts cluster based on color morph (Fig. 2). Silver and mottled males show sharp differences in the neuromolecular expression of transcripts (Fig. 2). Of the top 50 differentially expressed transcripts, 33 are up-regulated and 17 are down-regulated in mottled males compared to silver males (Fig. 3; Fig. S2, Table S2). Within the top 50 differentially expressed transcripts, two transcript variants each are detected for four genes: Rho GTPase activating protein 23a (*arhgap23a*), histone lysine methyltransferase complex subunit (*ash2l*), exotoses 1c (*ext1c*), and protein kinase C, epsilon a (*prkcea*; Fig. 3; Fig. S2, Table S2). Three transcripts are regulated in opposite directions for mottled males compared to silver males (*arhgao23a*, *ash2l*, and *ext1c*), while one transcript's variants are both up-regulated in mottled males (*prkcea*; Fig. 3; Fig. S2, Table S2).

For the top 50 differentially expressed transcripts, most molecular functions and biological processes are from gene ontology terms that were computationally inferred for Zebrafish based on phylogenetic similarity to organisms used in the experimental determination of protein function (Gaudet *et al*., 2011). Overall, transcripts separate into approximately 16 general biological functions (Table S3): metabolism (5 genes), neuron development and structure (6 genes), cell signaling (14 genes), development (4 genes), protein modification (10 genes), trafficking and cytoskeleton remodeling (9 genes), cell-cell adhesion (3 genes), cell migration (2 genes), DNA repair and replication (4 genes), transcription and translation (11 genes), chromatin and histone structure and modifications (4 genes), cell cycle (3 genes), apoptosis and autophagy (1 gene), synaptic transmission (3 genes), immunity (3 genes), and stress (1 gene). In general, synaptic transmission, cell signaling, trafficking, protein modification, and transcription and translation are up-regulated in mottled males compared to silver males.

Based on inferred functions, I identify five transcripts that are potentially important to the regulation of social behavior and include citations for their functions here. Genes involved in cell signaling and synaptic transmission are likely important to the regulation of social behavior. Growth factor pathways involve the down-regulated activin A receptor type 2A (*acvr2aa*; rev: Bilezikjian *et al.*, 2004) and up-regulated GIPC PDZ domain containing family, member 1 (*gipc1*; Lin *et al.*, 2006). The up-regulated transcripts calcium channel, voltage-dependent, gamma subunit 7a (*cacng7a*; Kato *et al.*, 2010), disks large homolog 4-like (*dlg4*; Chen *et al.*, 2011), and *gipc1* (Liu *et al.*, 2022) all regulate AMPA-type glutamate receptors. *gipc1* is also known to regulate dopamine receptor recruitment (Jeanneteau *et al.*, 2004b, 2004a) and melanin synthesis (Liu *et al.*, 2001; Kedlaya *et al.*, 2011). *acvr2aa* also regulates neural crest cell migration (Albertson *et al.*, 2005), which is involved in both pigmentation and brain development. The up-regulated transcripts kirre like nephrin family adhesion molecule 3b (*kirrel3b*) and *dlg4* are associated with social behavior in mice (Feyder *et al.*, 2010; Prince *et al.*, 2013).

These five transcripts were tested in a correlation matrix with social behavioral measures (Table 1). Because of the low sample size none of the differences in behaviors between male mosquitofish morphs can meet a significant P-value threshold via a Welch two-sample t-test (Fig. 4), however, the transcript *cacng7a* strongly correlates with aggression frequency (r = 0.89, P = 0.02; Fig.3, Table 1). *gipc1* correlates with aggression duration (r = 0.81, P = 0.05), while *kirrel3b* correlates with submission duration (r = 0.80, P = 0.05; Fig. 3; Table 1).

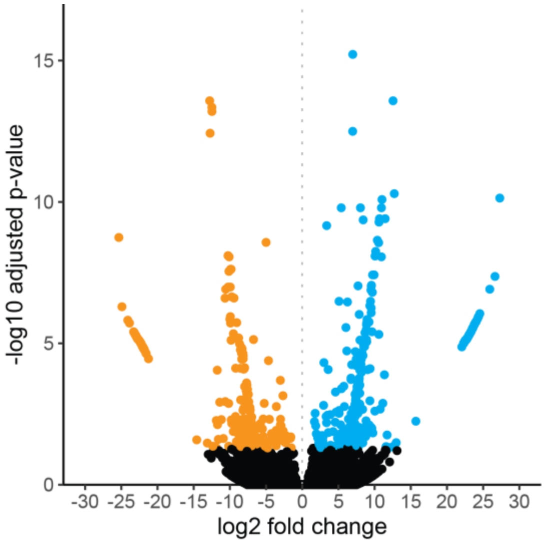

Figure 1. Volcano plot showing the differentially expressed genes in sampled male *G. holbrooki* color morphs. Transcripts that do not significantly differ in expression between morphs are in black. Transcripts that are down-regulated significantly in mottled males relative to silver males are orange, and up-regulated transcripts are blue. This volcano plot shows that a similar number of up- and down-regulated transcripts have statistically significant changes in log2fold expression.

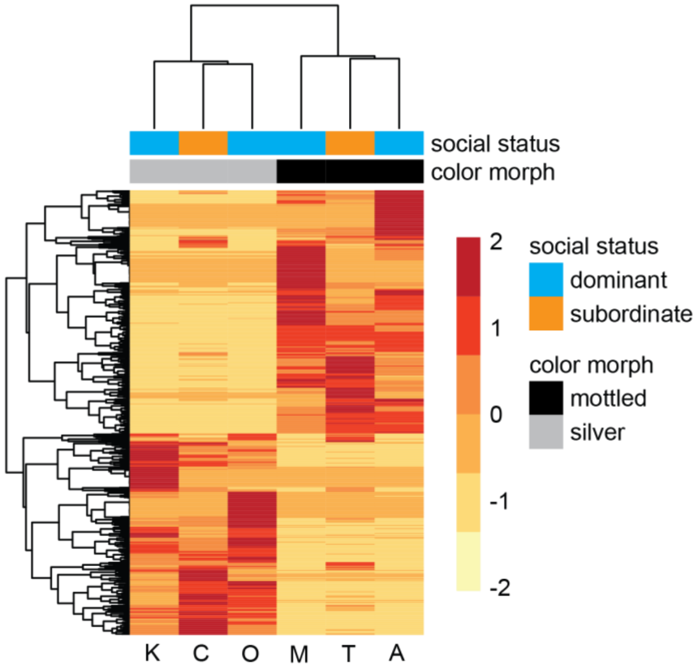

Figure 2. Heat map of the expression profiles for all differentially expressed genes of sampled male *G. holbrooki* color morphs. Expression is based on z-score and indicated by a gradient of red-orange-yellow color. Samples cluster sharply by color morph (mottled = black, silver = gray) instead of social status (dominant = blue, orange = subordinate). In the upper left quadrant, silver males show low expression of these transcripts. In the upper right quadrant, mottled males show the opposite response with high expression of these transcripts. Similarly, silver males have high transcript expression in the lower left quadrant, but mottled males have low transcript expression in the lower right quadrant.

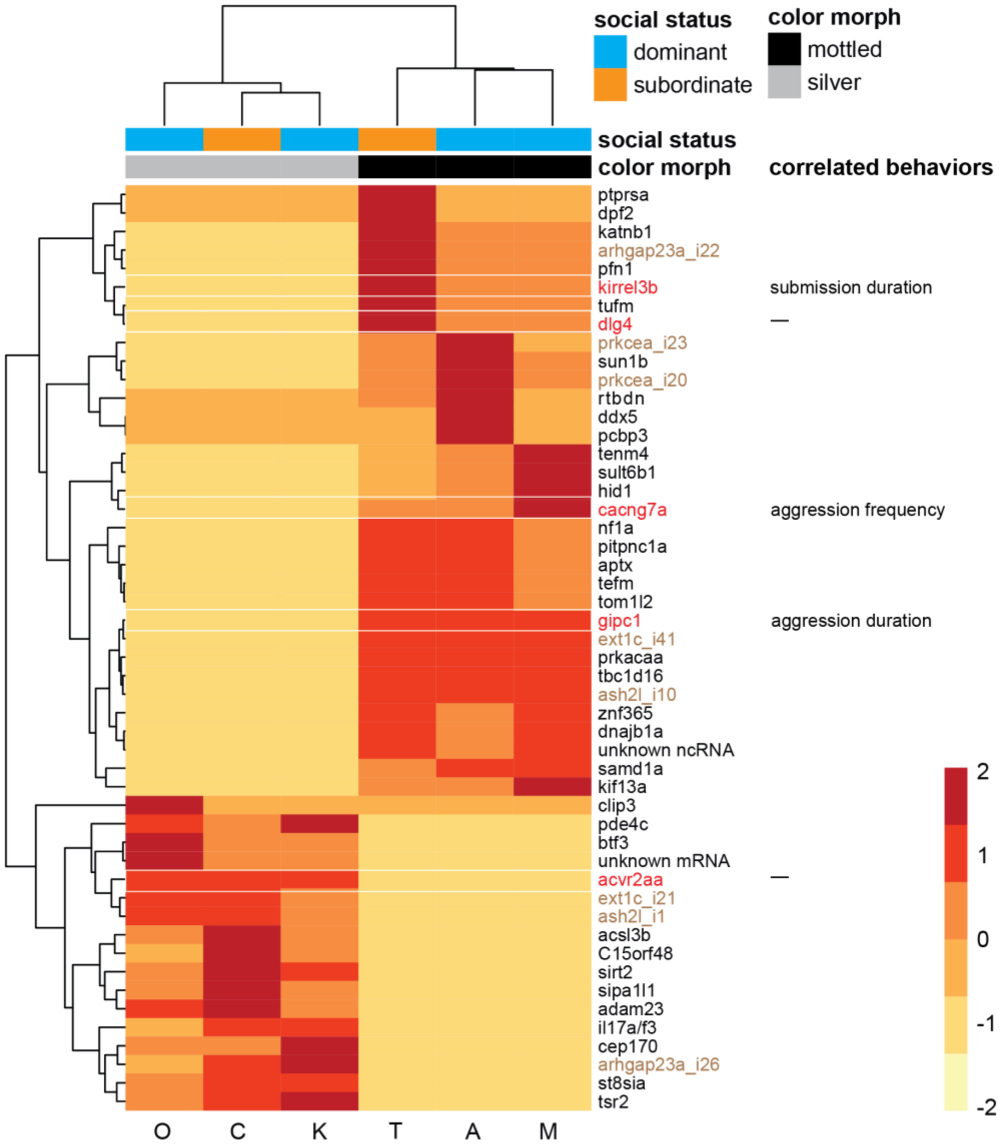

Figure 3. Heat map of the expression profiles for the top 50 differentially expressed genes of sampled male *G. holbrooki* morphs. Samples cluster sharply by color morph (mottled = black, silver = gray) instead of social status (dominant = blue, orange = subordinate). In the upper left quadrant, silver males show low expression of these transcripts. In the upper right quadrant, mottled males show the opposite response with high expression of these transcripts. Similarly, silver males have high transcript expression in the lower left quadrant, but mottled males have low transcript expression in the lower right quadrant. Two transcript variants each are detected for four genes (*arhgap23a*, *prkcea*, *ext1c*, and *ash2l*), and they are highlighted in brown. Transcript variants are labeled based on the isoform number (iX) in the transcriptome assembly. Three transcripts are regulated in opposite directions for mottled males compared to silver males (*arhgao23a*, *ash2l*, and *ext1c*), while one transcript's variants are both up-regulated in mottled males (*prkcea*). The five genes that potentially function in social behavior are highlighted in red, and white boxes demarcate their expression profiles (up-regulated in mottled males: *kirrel3b*, *dlg4*, *cacng7a*, *gipc1*; down-regulated in mottled males: *acvr2aa*). Correlated behaviors for these transcripts are next to them. *kirrel3b* correlates with submission duration and is most expressed in the subordinate mottled males (r = 0.80, P = 0.05). *cacng7a* and *gipc1* correlate with aggression frequency (r = 0.89, P = 0.02) and duration (r = 0.81, P = 0.05), respectively.

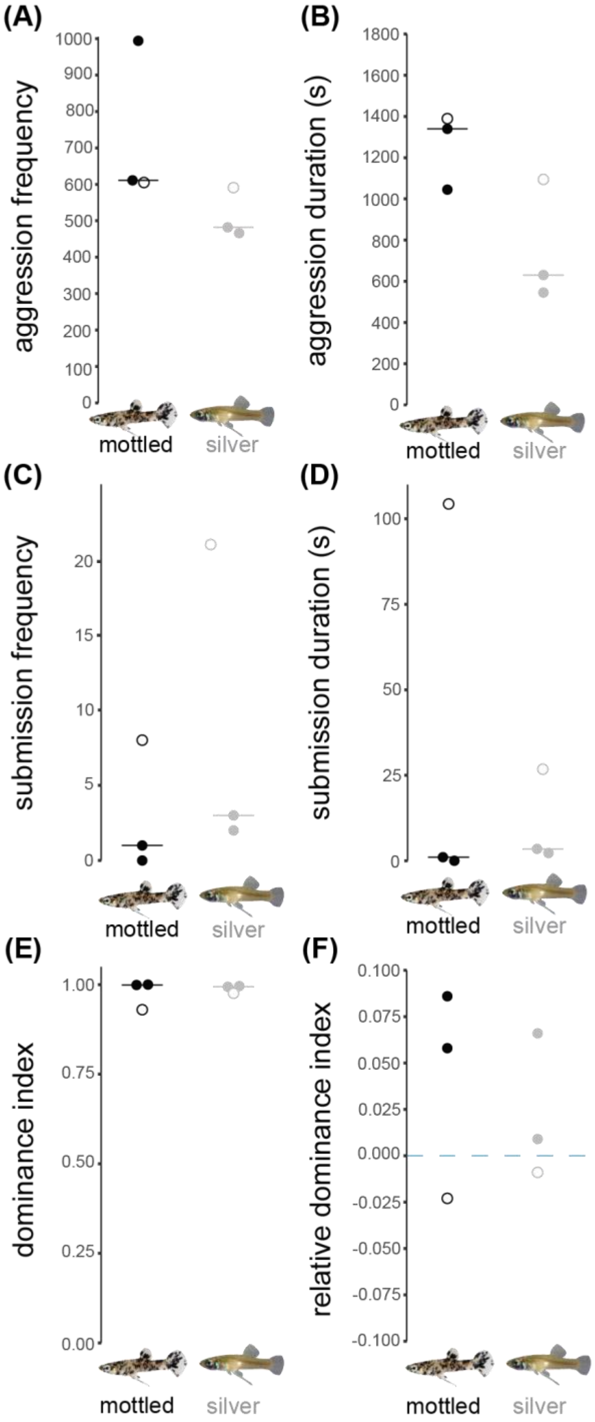

Figure 4. Social behaviors of sampled male *G. holbrooki* morph after a social interaction with a silver male. Mottled and silver morphs are black and grey, respectively. Dominant males are represented by closed circles, while subordinate males are open circles. Median lines are presented for each morph for **(A)** aggression frequency, **(B)** aggression duration, **(C)** submission frequency, **(D)** submission duration, and **(E)** dominance index. **(F)** For relative dominance index, a dashed blue line indicates when neither male in a social interaction is dominant. Morphs did not differ significantly in any of these behavioral measures (Welch two-sample t-test).

Table 1. Correlation matrix of five gene candidates with male *G. holbrooki* social behavior. Based on gene ontology terms, five transcripts potentially regulate social behavior. These transcripts are tested in a correlation matrix against six measures of male mosquitofish social behavior (aggression frequency and duration, submission frequency and duration, dominance index, and relative dominance index). Pearson correlation coefficients and associated P-values (in parentheses) are presented. Significant correlations are highlighted in bold. The transcript *cacng7a* strongly correlates with aggression frequency. *gipc1* correlates with aggression duration, while *kirrel3b* correlates with submission duration.

	acvr2aa	*cacng7a*	*dlg4*	*gipc1*	*kirrel3b*
aggression frequency	-0.60 (0.20)	**0.89** **(0.02)**	0.31 (0.54)	0.72 (0.10)	0.22 (0.68)
aggression duration (s)	-0.70 (0.12)	0.69 (0.13)	0.74 (0.09)	**0.81** **(0.05)**	0.71 (0.11)
submission frequency	0.50 (0.31)	-0.50 (0.31)	-0.20 (0.71)	-0.39 (0.44)	-0.11 (0.84)
submission duration (s)	-0.29 (0.58)	-0.03 (0.96)	0.68 (0.14)	0.32 (0.54)	**0.80** **(0.05)**
dominance index	0.21 (0.69)	0.10 (0.85)	-0.62 (0.19)	-0.24 (0.65)	-0.75 (0.09)
relative dominance index	-0.30 (0.57)	0.54 (0.27)	-0.16 (0.76)	0.25 (0.63)	-0.30 (0.56)

5.4 Discussion

Based on the biological processes identified for the top 50 differentially expressed transcripts, major changes exist in cytoskeleton remodeling, transcription, cell signaling, and synaptic transmission, which are likely related to changes in neurons as males responded to social stimuli and adjust the behavioral response. Following complete annotation of the transcriptome, functional enrichment analyses using gene ontology terms for all differentially expressed genes will determine how these molecular functions and biological processes differ between male mosquitofish color morphs. Of the top 50 differentially expressed transcripts, five are identified as potentially relating to social behavior: the activin A receptor type 2A (*acvr2aa*), calcium channel, voltage-dependent, gamma subunit 7a (*cacng7a*), disks large homolog 4-like (*dlg4*), GIPC PDZ domain containing family, member 1 (*gipc1*), and kirre like nephrin family adhesion molecule 3b (*kirrel3b*). The transcripts *cacng7a* and *gipc1* correlate with aggression frequency and duration, respectively, while *kirrel3b* correlates with submission duration. Following complete transcriptome annotation, additional differentially expressed transcripts related to social behavior may be identified.

The activin receptor *acvr2aa* is down-regulated in mottled males compared to silver males. The gene *bambia* (BMP and activin membrane-bound inhibitor) encodes a protein that inhibits ACVR2AA and was down-regulated in dominant male Nile tilapia (*Oreochromis niloticus)* (Thönnes *et al*., 2022), meaning the receptor is likely activated in brains of socially dominant tilapia. Given that mottled males usually dominate silver males, it is surprising to see down-regulation of this receptor by mottled mosquitofish. *acvr2aa* is part of the *TGF-β* (transforming growth factor β) signaling pathway, through which activin affects the production of gonadal and pituitary hormones (rev: Bilezikjian *et al*., 2004). Specifically, activin stimulates follicle stimulating hormone (FSH) production, but suppresses growth hormone (GH) and adrenocorticotropic hormone (ACTH) production. If *acvr2aa* is down-regulated in mottled males, the production of both GH and ACTH would be predicted to increase, which in turn may increase cortisol, androgens, β-endorphins, adrenaline, and noradrenaline levels (rev: Bornstein and Chrousos, 1999), which have been previously correlated with social behavior and/or melanin-based coloration. High levels of circulating sex steroids are associated with both the expression of melanin-based coloration and higher social dominance (rev: Oliveira *et al*., 2002; San-Jose and Roulin, 2018). β-endorphins are elevated in the amygdala during social defeat in monogolian gerbils (*Meriones unguiculatus*; Raab *et al*., 1985). Although mottled males usually dominate silver males, elevated β-endorphins could still be important in the regulation of social conflict outcomes for mottled males. Although stickleback (*Gasterosteus aculeatus*) that encountered an intruder were highly aggressive and had more circulating noradrenaline (Bell *et al*., 2007), noradrenaline is known to aggregate melanosomes in fish (rev: Karlsson *et al*., 1987), which would not explain the black blotches of mosquitofish morphs. Higher corticotrophin-release hormone (CRH) is also associated with proopiomelanocortin (POMC) transcription, which initiates melanin synthesis and melanocyte differentiation (rev: Aguilera, 1994). Finally, activin and the *TGF-β* pathway normally inhibit melanocyte differentiation (Bilezikjian *et al*., 1991; Moustakas, 2008), so down-regulation of *acvr2aa* may directly release this inhibition and stimulate melanin synthesis and melanocyte differentiation. Thus, *acvr2aa* and the *TGF-β* pathway are a potential candidate as a molecular system underlying the correlation of pigmentation and social behavior in male mosquitofish morphs.

The calcium channel *cacng7a* is up-regulated in mottled morphs and correlated with aggression frequency. This calcium channel positively regulates AMPA-type glutamate receptors (Kato *et al*., 2010), which have a strong correlation with social dominance in mice (Park *et al*., 2018). In both dominant and subordinate male Syrian hamsters (*Mesocricetus auratus*), the AMPA-glutamate receptor *gria2* (glutamate ionotropic receptor AMPA type subunit 2) was up-regulated in the basolateral amygdala following acute social conflict (McCann *et al*., 2019), indicating that these receptors are an important part of processing social interactions. Specifically, mice deficient in AMPA-type glutamate receptors had reduced aggression (Vekovischeva *et al*., 2004), so up-regulation of *cagng7a* may act through these receptors to increase aggression frequency in mottled males. Furthermore, AMPA-type glutamate receptors are known to promote the differentiation of melanocytes and stabilize their morphology (Hoogduijn *et al.*, 2006). The significant correlation of *cacgn7a* expression with aggression frequency and the mottled color pattern demonstrates that the glutamate system is a strong potential candidate for pleiotropic effects on melanin-based pigmentation and social behavior in male mosquitofish.

Mottled males up-regulate *dlg4*, which encodes PSD-95 (postsynaptic density protein 95), also called SAP-90 (synapse-associated protein 90), that acts as part of a postsynaptic complex that binds to both NMDA- and AMPA-type glutamate receptors (Chen *et al.*, 2011; Jiang-Xie *et al.*, 2014; Gao and Mack, 2021). Disruption of *dlg4* is associated with changes in social behavior of mice that mimic those in autism spectrum disorder (Gao and Mack, 2021). DLGAP2 (DLG Associated Protein 2) forms part of the postsynaptic complex with PSD-95; defects in DLGAP2 were associated with higher aggression and social dominance in mice (Jiang-Xie *et al.*, 2014), suggesting that it may lower aggression and social dominance when expressed. However, the transcript does not correlate with any specific behavioral measures, so its effect on male mosquitofish behavior requires further study. Nevertheless, the up-regulation of *dlg4* once again identifies the glutamate system as potentially important in the regulation of social behaviors correlated with color morph in male mosquitofish.

Mottled males also up-regulate *kirrel3b*, a transcript for a kirre-like protein, important in synaptic adhesion and creation (Taylor *et al.*, 2020). Mice knockouts for this protein exhibited deficits in social behavior, communication, and identification of familiar social stimuli, similar to changes associated with autism spectrum disorder (Hisaoka *et al.*, 2018; Taylor *et al.*, 2020). *kirrel3b* correlates with submission duration, but, to my knowledge, no studies have investigated this protein’s effect on submissive behavior. Furthermore, I could find no association between this gene transcript and pigmentation. Up-regulation of this transcript may be part of a stronger general response to social interactions by mottled male mosquitofish, and perhaps is important in the perception, processing, and response to socially relevant cues.

Finally, mottled males up-regulate the transcript *gipc1*, which was also correlates with aggression duration. An allele of this gene was previously associated with the presence of the melanic-side spotting pattern in *G. holbrooki* (Kottler *et al*., 2020). *gipc1* functions in vesicle trafficking and sorting proteins for pigment synthesis to melanosomes within melanocytes (Liu *et al*., 2001). This protein also may be important for targeting receptors during processes involved in synaptic transmission, such as retrograde transport and recycling of neurotransmitters and localization of axons and dendrites for synapse formation (Lee *et al*., 2001). Thus, *gipc1* could be important in the regulation of synaptic transmission during aggressive social interactions in mottled male mosquitofish as well as pigment synthesis and aggregation. For example, GIPC1 acts as a scaffolding protein for the recruitment of GTPase activating proteins by the dopamine 2 receptor (D2R); GIPC and D2R are often co-expressed (Jeanneteau *et al*., 2004b). D2R is associated with a decrease in social dominance in primates (Yamaguchi *et al*., 2017), which is unexpected given that mottled males usually dominate silver males. However, the effect of D2R on affiliative behavior depended on social context in the cichlid fish *Neolamprologus pulcher* (Antunes *et al*., 2022), so the effect of D2R on dominance may depend on both social environment and species. Other neuromodulators like the nonapeptides arginine vasotocin and oxytocin, are known to affect aggression and affiliative behaviors differently in voles and mice depending on the species' social system (rev: Goodson, 2008). Taken together, it is possible that the up-regulation of *gipc1* leads to an increase in functional D2R, which in turn increases affiliative behavior. An increase in affiliative behavior would explain the correlation of *gipc1* with increased aggression duration, which is also slightly higher in mottled males, as mottled males may be spending more time focused on these social interactions than silver males. Given its previous identification as a sex-linked gene that affects the presence of the mottled color pattern in *G. holbrooki*, its correlation with aggression frequency, and its known functions in melanin synthesis and dopamine receptor recruitment, *gipc1* is a prime candidate for the pleiotropic regulation of color pattern and social behavior in male Eastern Mosquitofish morphs.

Male Eastern Mosquitofish (*G. holbrooki*) exhibited distinct, morph-specific neuromolecular responses to acute social interaction. These 563 differentially expressed transcripts covered a wide variety of molecular functions and biological processes. Whole-brain gene expression of Western honey bees (*Apis mellifera*) occurred in waves following aggression against intruders (Shpigler *et al*., 2017). Their expression was initially characterized by cytoskeleton remodeling, and later by genes related to transcription factors and hormones. Similar biological processes from both of these gene expression waves were identified for the top 50 differentially expressed transcripts. If a similar temporal pattern of gene expression exists in *G. holbrooki*, the time point chosen for this acute social interaction may be at a transition point between the two neuromolecular responses. Thus, compared to this study, investigating neuromolecular responses for male mosquitofish morphs that experienced a longer period of social interaction, such as in stable dominance hierarchies, may reveal more transcripts or different transcripts related to neurochemical signaling pathways that affect social dominance.

In conclusion, the differential responses in neuromolecular expression patterns between male *G. holbrooki* color morphs allowed the identification of five candidate genes that potentially regulate differences in social behavior between morphs. GIPC1 and the molecular systems for *TGF-β* and glutamate were identified as strong candidates for the correlation of melanin-based pigmentation and social behavior. Future studies should use immunohistochemistry or *in situ* hybridization to identify where in the mosquitofish brain these genes are differentially expressed between morphs, followed by experimental testing of how the protein affects pigmentation and social behavior in mosquitofish through pharmacological and/or genetic manipulations.

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5.6 Appendix

Table S1. Sample overview for mottled and silver male mosquitofish used in this study. Color morph (silver = s, mottled = m) and social status (dom = dominant, sub = subordinate) are listed for each male mosquitofish. RNA purity (260/280 and 260/30 absorbance ratios from Nanodrop), concentration (ng/μL) as measured by Qubit, and the RNA integrity number (RIN) from Agilent Bioanalyzer are provided. Incorrect initial volumes were used during RIN assessment that results in failure to obtain values for some samples, but visual inspection of the electrophoretic traces confirmed that all samples had maintained high RNA integrity. This table also shows the raw, trimmed, and mapped number of sequences for each sample.

sample	**morph**	**status**	**260/280**	**260/230**	**ng/μL**	**RIN**	**raw**	**trimmed**	**mapped**
C	s	sub	2.32	1.92	17.1	7.40	21,199,525	20,659,776	16,405,554
K	s	dom	2.21	2.12	17.1	7.70	25,031,358	24,199,805	20,005,571
O	s	dom	1.99	1.82	21.1	N/A	20,017,148	19,340,887	15,118,099
A	m	dom	2.19	2.14	30.7	9.50	22,193,315	21,507,984	17,861,677
M	m	dom	2.36	2.21	20.4	N/A	19,959,017	19,494,400	15,854,810
T	m	sub	2.15	2.07	64.7	N/A	21,925,387	21,249,179	17,875,975

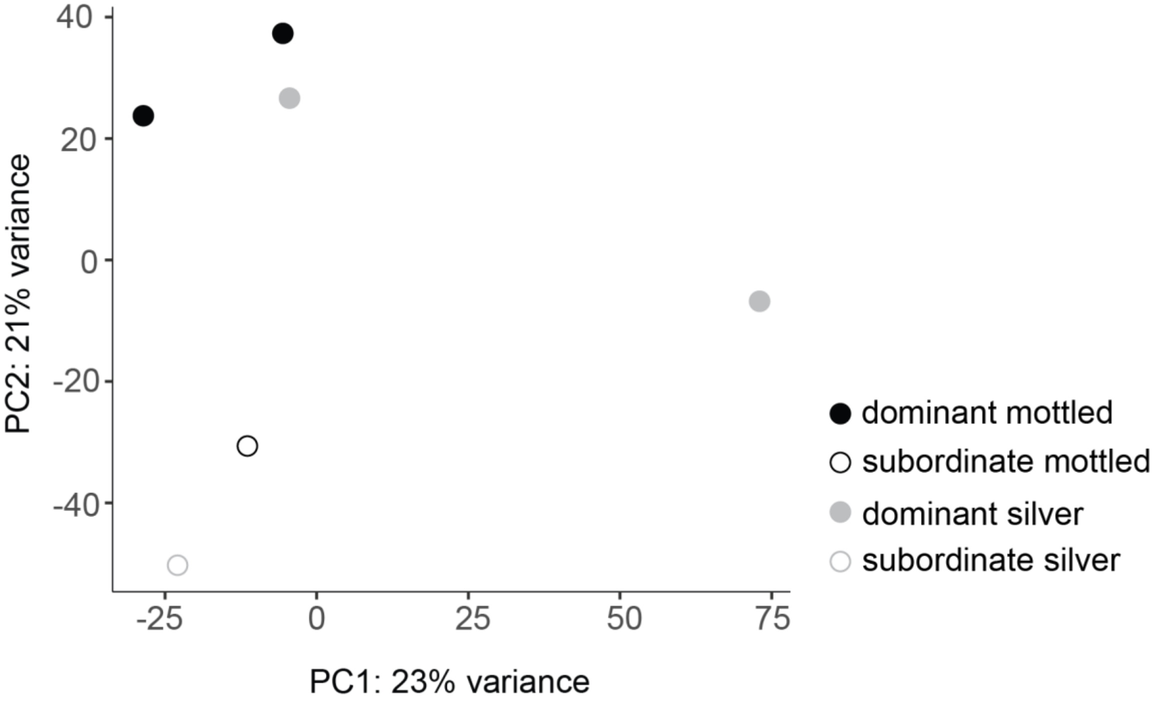

Figure S1. Principal Components Analysis plot to cluster samples by expression profiles for all genes. Mottled and silver males do not cluster sharply by color morph, however, dominant mottled males do cluster together in the upper left quadrant. Outliers in the bottom left quadrant are subordinate males of both color morphs. Initial clustering of samples is best described with both color morph and social dominance status, so the model for differential gene expression is specified as ~ social status + color morph.

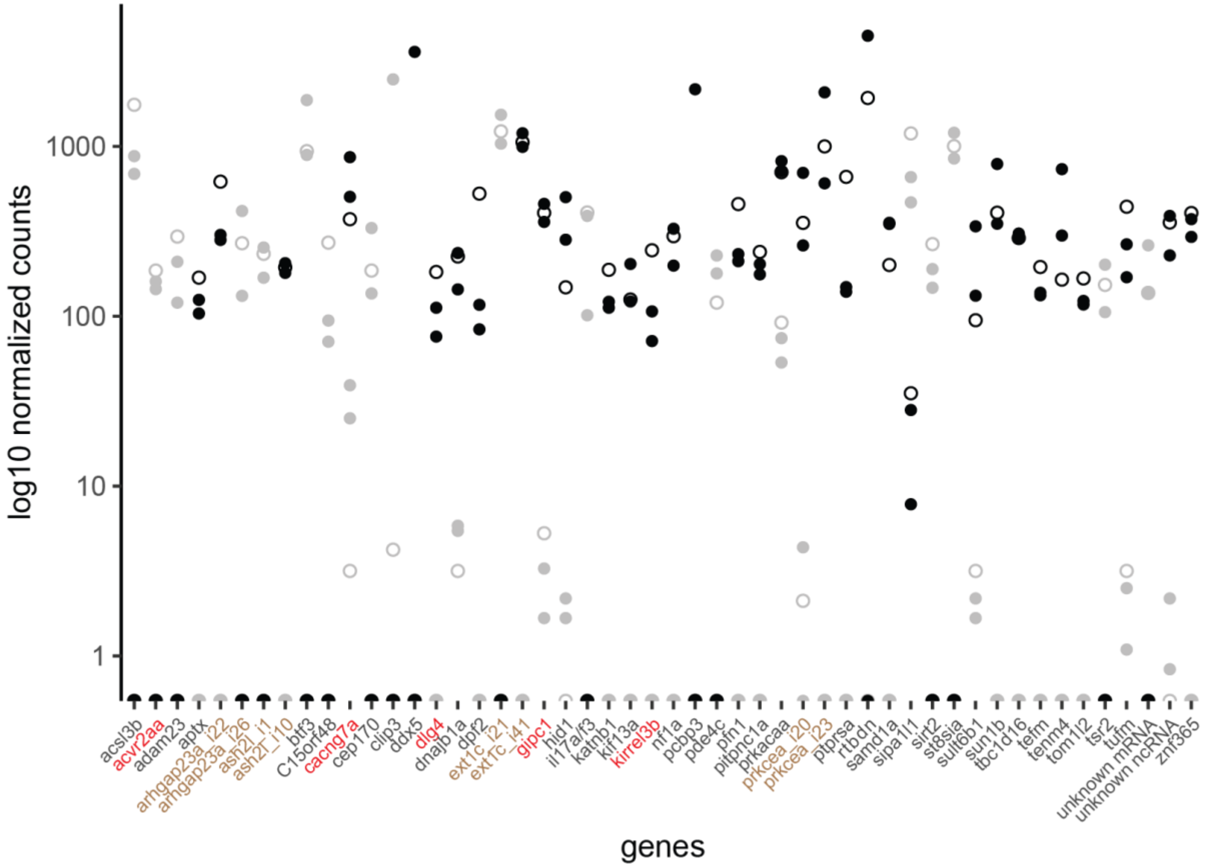

Figure S2. Plot of log10 normalized counts for the top 50 differentially expressed genes between male mosquitofish color morphs. Color indicates color morph (mottled = black, silver = grey), while shape indicates social status (dominant = closed circle, subordinate = open circle). Two transcript variants each are detected for four genes, and they are highlighted in brown (*arhgap23a*, *prkcea*, *ext1c*, and *ash2l*). Transcript variants are labeled based on the isoform number (iX) in the transcriptome assembly. Three transcripts are regulated in opposite directions for mottled males compared to silver males (*arhgao23a*, *ash2l*, and *ext1c*), while one transcript's variants are both up-regulated in mottled males (*prkcea*). The five transcripts that potentially function in social behavior are highlighted in red (up-regulated in mottled males: *kirrel3b*, *dlg4*, *cacng7a*, *gipc1*; down-regulated in mottled males: *acvr2aa*). *kirrel3b* correlates with submission duration (r = 0.80, P = 0.05). *cacng7a* and *gipc1* correlate with aggression frequency (r = 0.89, P = 0.02) and duration (r = 0.81, P = 0.05), respectively.

Table S2. The top 50 differentially expressed genes between male mosquitofish color morphs. Gene abbreviation, gene full name, and whether the transcripts are up-regulated or down-regulated in mottled males compared to silver males are shown. 33 transcripts are up-regulated and 17 are down-regulated in mottled males compared to silver males. Transcript variants are labeled based on the isoform number (iX) in the transcriptome assembly. Three transcripts are regulated in opposite directions for mottled males compared to silver males (*arhgao23a*, *ash2l*, and *ext1c*), while one transcript's variants are both up-regulated in mottled males (*prkcea*).

gene	**name**	**regulation**
acsl3b	acyl-CoA synthetase long chain family member 3b	down
acvr2aa	activin A receptor type 2A	down
adam23	disintegrin and metalloproteinase domain-containing protein 23	down
arhgap23a_i26	Rho GTPase activating protein 23a, transcript variant i26	down
ash2l_i1	histone lysine methyltransferase complex subunit, transcript variant i1	down
btf3	basic transcription factor 3	down
C15orf48	Chromosome 15 Open Reading Frame 48	down
cep170	centrosomal protein 170	down
clip3	CAP-GLY domain containing linker protein 3	down
ext1c_i21	exostoses 1c, transcript variant i21	down
il17a/f3	interleukin 17a/f3	down
pde4c	phosphodiesterase 4c	down
sipa1l1	signal-induced proliferation-associated 1-like protein 1	down
sirt2	sirtuin 2 (silent mating type information regulation 2)	down
st8sia	sia-alpha-2,3-Gal-beta-1,4-GlcNAc-R:alpha 2,8-sialyltransferase-like	down
tsr2	TSR2 ribosome maturation factor	down
unknown mRNA	uncharacterized mRNA	down
aptx	aprataxin	up
arhgap23a_i22	Rho GTPase activating protein 23a, transcript variant i22	up
ash2l_i10	histone lysine methyltransferase complex subunit, transcript variant i10	up
cacng7a	calcium channel, voltage-dependent, gamma subunit 7a	up
ddx5	DEAD (Asp-Glu-Ala-Asp) box helicase 5	up
dlg4	disks large homolog 4-like	up
dnajb1a	DnaJ heat shock protein (Hsp40) member B1a	up
dpf2	zinc and double PHD fingers family 2	up
ext1c_i41	exostoses 1c, transcript variant i41	up
gipc1	GIPC PDZ domain containing family, member 1	up
hid1	HID1 domain containing	up
katnb1	katanin p80 (WD repeat containing) subunit B 1	up
kif13a	kinesin family member 13A	up
kirrel3b	kirre like nephrin family adhesion molecule 3b	up
nf1a	neurofibromin 1a	up

pcbp3	poly(rC)-binding protein 3	up
pfn1	profilin 1	up
pitpnc1a	phosphatidylinositol transfer protein cytoplasmic 1a	up
prkacaa	protein kinase, CAMP-dependent, catalytic, alpha, genome duplicate a	up
prkcea_i20	protein kinase C, epsilon a, transcript variant i20	up
prkcea_i23	protein kinase C, epsilon a, transcript variant i23	up
ptprsa	protein tyrosine phosphotase receptor type Sa	up
rtbdn	retbindin	up
samd1a	sterile alpha motif domain containing 1a	up
sult6b1	sulfotransferase family, cytosolic, 6b, member 1	up
sun1b	Sad1 and UNC84 domain containing 1b	up
tbc1d16	TBC1 domain family, member 16	up
tefm	transcription elongation factor, mitochondrial	up
tenm4	teneurin transmembrane protein 4	up
tom1l2	TOM1-like protein 2	up
tufm	Tu translation elongation factor, mitochondrial	up
unknown ncRNA	uncharacterized non-coding RNA	up
znf365	zinc finger protein 365	up

Table S3. Molecular function(s) and biological process(es) for the top 50 differentially expressed genes between color morphs based on gene ontology terms. Gene ontology terms are based on curated annotations from the literature and computational inferences (Ashburner *et al*., 2000; Hill *et al*., 2008; The Gene Ontology Consortium, 2021). Transcript functions are identified through their gene ontology terms listed in the 2023_01 release of the UniprotKB database (The UniProt Consortium, 2023). Where possible, closely related fish species and the Zebrafish (*Danio rerio*) were used to determine function. If the annotation was not available for fish, mouse (*Mus musculus*) or human (*Homeo sapiens*) were used. A + or - indicates that the protein positively or negatively regulates that process, respectively. The five transcripts that potentially function in social behavior are bolded (up-regulated in mottled males: *kirrel3b*, *dlg4*, *cacng7a, gipc1*; down-regulated in mottled males: *acvr2aa*).

gene	molecular function	biological process
acsl3b	long-chain fatty acid-CoA ligase activity	long-chain fatty acid metabolic process long-chain fatty-acyl-CoA metabolic process neuron differentiation
acvr2aa	activin binding activin receptor activity ATP binding metal ion binding protein serine/threonine kinase activity	activin receptor signaling pathway cartilage development cellular response to growth factor stimulus hindbrain development neural crest cell migration protein phosphorylation
adam23	metalloendopeptidase activity	cell adhesion cellular response to leukemia inhibitory factor proteolysis
arhgap23a_i26	GTPase activator activity	signal transduction
ash2l_i1	beta-catenin binding metal ion binding transcription cis-regulatory region binding	cellular response to DNA damage stimulus histone H3-K4 methylation + cell population proliferation + transcription by RNA polymerase II response to estrogen transcription initiation-coupled chromatin remodeling

btf3	RNA binding	- protein localization to endoplasmic reticulum protein transport
C15orf48	membrane protein	N/A
cep170	microtubule organization	centriole subdistal appendage assembly
clip3	microtubule plus-end binding	cytoplasmic microtubule organization
ext1c_i21	acetylglucosaminyltransferase activity glucuronosyl-N-acetylglucosaminyl-proteoglycan 4-alpha-N-aceterylglucosaminyltransferase activity glucuronosyltransferase activity glycosyltransferase activity metal ion binding N-acetylglucosaminyl-proteoglycan 4-beta-glucuronosyltransferase activity	heparan sulfate proteoglycan biosynthetic process protein glycosylation
il17a/f3	cytokine activity	cellular response to lippolysaccharide inflammatory response
pde4c	3’,5’-cyclic-AMP phosphodiesterase activity 3’,5’-cyclic-nucleotide phosphodiesterase activity metal ion binding	cAMP catabolic process - insulin secretion involved in cellular response to glucose stimulus signal transduction
sipa1l1	actin filament binding ephrin receptor binding GTPase activator activity protein kinase binding protein-containing complex binding ubiquitin protein ligase binding	actin cytoskeleton organization axonogenesis dendrite morphogenesis dendritic spine morphogenesis GTPase activity postsynaptic density assembly synaptic plasticity
sirt2	histone deacetylase activity metal ion binding NAD+ binding NAD-dependent histone deacetylase activity NAD-dependent histone H4K16 deacetylase activity	cellular response to caloric restriction cellular response to hypoxia cellular response to oxidative stress cilium assembly hemopoeisis

	NAD-dependent protein deacetylase activity NAD-dependent protein demyristoylase activity NAD-dependent protein depalmitoylase activity tubulin deacetylase activity	- angiogenesis - autophagy - cell population proliferation - oligodendrocyte progenitor proliferation - protein catabolic process - reactive oxygen species metabolic process -/+ transcription by RNA polymerase II + DNA binding + execution phase of apoptosis + proteasomal ubiquitin-dependent protein catabolic process protein deacetylation regulation of cell cycle tubulin deacetylation
st8sia	sialyltransferase activity	protein glycosylation
tsr2	pre-rRNA processing	maturation of SSU-rRNA from tricstronic rRNA transcript
unknown mRNA	N/A	N/A
aptx	DNA 5’-adenosine monophosphate hydrolase activity DNA 3’-diphosho-5’-guanosine diphosphatase double-stranded RNA binding metal ion binding mismatched DNA binding single-stranded DNA binding	double-strand break repair single-strand break repair
arhgap23a_i22	GTPase activator activity	signal transduction
ash2l_i10	beta-catenin binding metal ion binding transcription cis-regulatory region binding	cellular response to DNA damage stimulus histone H3-K4 methylation + cell population proliferation + transcription by RNA polymerase II

		response to estrogen transcription initiation-coupled chromatin remodeling
cacng7a	channel regulator activity voltage-gated calcium channel activity	neurotransmitter receptor internalization neurotransmitter receptor transport, postsynaptic endosome to lysosome + synaptic transmission, glutamatergic postsynaptic neurotransmitter receptor diffusion trapping regulation of AMPA receptor activity transmission of nerve impulse
ddx5	ATP binding hydrolase activity nucleic acid binding RNA helicase activity	RNA helicase
dlg4	protein heterodimerization activity	AMPA glutamate receptor clustering cell-cell adhesion chemical synaptic transmission postsynaptic neurotransmitter receptor diffusion trapping receptor clustering receptor localization to synapse social behavior synapse organization
dnajb1a	chaperone binding Hsp70 protein binding transcription corepressor activity unfolded protein binding	chaperone cofactor-dependent protein refolding lens development in camera-type eye - transcription by RNA polymerase II
dpf2	histone binding metal ion binding transcription coregulator activity	- DNA-templated transcription nervous system development + transcription by RNA polymerase II

ext1c_i41	acetylglucosaminyltransferase activity glucuronosyl-N-acetylglucosaminyl-proteoglycan 4-alpha-N-aceterylglucosaminyltransferase activity glucuronosyltransferase activity glycosyltransferase activity metal ion binding N-acetylglucosaminyl-proteoglycan 4-beta-glucuronosyltransferase activity	heparan sulfate proteoglycan biosynthetic process protein glycosylation
gipc1	actin binding GTPase activator activity identical protein binding myosin binding PDZ domain binding signaling receptor binding	cellular response to interleukin-Y chemical synaptic transmission endothelial cell migration G protein-coupled receptor signaling pathway glutamate secretion - proteasomal ubiquitin-dependent protein catabolic process + cytokinesis + melanin biosynthetic process + transforming growth factor beta receptor signaling pathway presynaptic modulation of chemical synaptic transmission protein targeting protein stability synaptic plasticity
hid1	protein biosynthesis and vesicle formation	involved in cancers
katnb1	microtubule binding	cell cycle cell division microtubule severing
kif13a	ATP binding microtubule binding microtubule motor activity	microtubule-based movement system development
kirrel3b	cell adhesion molecule binding	cell-cell adhesion hemopoiesis hippocampus development

		homophilic cell adhesion via plasma membrane adhesion molecules neuron migration neuron projection morphogenesis principal sensory nucleus of trigeminal nerve development social behavior synapse assembly
nf1a	GTPase activator activity	GTPase activity
pcbp3	mRNA 3’-UTR binding mRNA binding single-stranded DNA binding	gene expression RNA metabolic process
pfn1	actin binding	actin cytoskeleton organization convergent extension involved in gastrulation epiboly involved in gastrulation with mouth forming second + actin filament bundle assembly actin filament polymerization
pitpnc1a	phosphatidylinositol binding phosphatidylinositol transfer activity	phosphatidylinositol metabolic process phospholipid transport
prkacaa	ATP binding cAMP-dependent protein kinase activity	protein phosphorylation
prkcea_i23	ATP binding protein kinase C activity	animal organ development cell development protein phosphorylation
prkcea_i22	ATP binding protein kinase C activity	animal organ development cell development protein phosphorylation
ptprsa	protein tyrosine phosphatase activity	protein dephosphorylation

rtbdn	riboflavin binding riboflavin transmembrane transporter activity signaling receptor activity	retinal flavin transport
samd1a	chromatin binding histone binding	- DNA-templated transcription
sult6b1	sulfotransferase activity	cellular response to xenobiotic stimulus sulfation
sun1b	protein-membrane adaptor activity	nuclear envelope organization
tbc1d16	GTPase activator activity	activation of GTPase activity
tefm	DNA polymerase processivity factor activity nucleic acid binding	mitochondrial transcription oxidative phosphorylation transcription elongation by mitochondrial RNA polymerase
tenm4	cell adhesion molecule binding protein heterodimerization activity protein homodimerization activity	axon guidance central nervous system myelin formation heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules homophilic cell adhesion via plasma membrane adhesion molecules neuron development + gastrulation + myelination + neuron projection development + oligodendrocyte differentiation signal transduction
tom1l2	clathrin binding phosphatidylinositol binding ubiquitin binding	protein transport signal transduction
tufm	GTP binding GTPase activitiy	translational elongation

	translation elongation factor activity	
unknown ncRNA	N/A	N/A
znf365	lipid antigen binding lipopolysaccharide binding	defense response to Gram-negative bacterium regulation of DNA strand resection involved in replication fork processing regulation of double-strand break repair via homologous recombination regulation of neuron projection development telomere maintenance

VITA

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2010-2014	B.Sc., Honors, Animal Biology University of Alberta Edmonton, Alberta, Canada
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SELECTED PUBLICATIONS, PRESENTATIONS, AND GRANTS

DuVal M, Gilbert M, Watson D, Zerulla T, Tierney K, Allison W (2014) Growth Differentiation Factor 6 as a putative risk factor in neuromuscular degeneration. PLoS One. 9: e89183.

Gilbert M, Zerulla T, Tierney K (2014) Zebrafish (*Danio rerio*) as a model for the study of aging and exercise: physical ability and trainability decreases with age. Experimental Gerontology. 50:106-113.

Reichert M, Blunt B, Gabruch T, Zerulla T, Ralph A, Gamal El-Din M, Sutherland B, Tierney K (2017) Sensory and behavioral responses of a model fish to oil sands process-affected water with and without treatment. Environmental Science and Technology. 51(12): 7128-7137.

Society for Integrative and Comparative Biology (SICB) Grants-in-Aid-of-Research. (2017) $1000

FIU Biomedical Research Initiative Student Summer Research Award. (2017) $2910

Sigma Xi Grants-in-Aid-of-Research. (2018) $1000

Zerulla T, Stoddard P (2018) Social behavior differences between males exhibiting a color polymorphism in the Eastern Mosquitofish (*Gambusia holbrooki*). SICB Annual Meeting. Poster.

Zerulla T, Vallori J, Stoddard P (2018) Social behavior differences between males exhibiting a color polymorphism in the Eastern Mosquitofish (*Gambusia holbrooki*). Biological Research Symposium, FIU. Poster. 2nd Place Award.

Zerulla T, Vallori J, Stoddard P (2018) A model for phenotypic covariation: color-behavior polymorphism in male Eastern Mosquitofish (*Gambusia holbrooki*). Annual Biomedical and Comparative Immunology Symposium, FIU. Oral presentation.

Zerulla T (2018) Why do melanistic Eastern Mosquitofish (*Gambusia holbrooki*) exist? South Florida Natural Resource Center (SFNRC) Seminar - National Parks Service. Invited oral presentation.

Zerulla T (2019) Undergraduate lab module investigating how hurricanes affect Florida's bird biodiversity using iNaturalist data. 5th Life Discovery – Doing Science Education Conference. Oral presentation.

Zerulla T, Stoddard P (2019) Melanistic and silver Eastern Mosquitofish (*Gambusia holbrooki*) males respond differently to opponent size and color. 8th Conference of Poeciliid Biologists. Poster.

Zerulla T, Stoddard P (2020) Color morphs of Eastern Mosquitofish respond differently to socially relevant cues. Biological Research Symposium, FIU. Oral presentation.

Zerulla T (2020) A new animal model for social cue disorders. FIU: 3 Minute Thesis Competition. Oral presentation. 1st Place Award and People's Choice Award.

Zerulla T, Stoddard P (2020) Eastern Mosquitofish (*Gambusia holbrooki*) color morphs respond differently to socially relevant cues. Animal Behavior Society Virtual Conference. Oral presentation.

Zerulla T, Stoddard P (2020) Eastern Mosquitofish (*Gambusia holbrooki*) color morphs respond differently to socially relevant cues. Sigma Xi Virtual Annual Meeting and Student Research Conference. Poster.

Zerulla T, Stoddard P (2021) The biology of polymorphic melanic side-spotting patterns in poeciliid fishes. Frontiers in Ecology and Evolution. 8:477.

Zerulla T, Stoddard P (2021) Eastern Mosquitofish (*Gambusia holbrooki*) color morphs respond differently to socially relevant cues. Virtual Poeciliid Forum. Oral presentation.

Zerulla T (2021) TikTok on the clock: science communication in the age of social media. Biological Research Symposium, FIU. Oral presentation.

Zerulla T, Stoddard P (2021) Eastern Mosquitofish (*Gambusia holbrooki*) color morphs respond differently to socially relevant cues. Graduate Student Appreciation Week, FIU. 1st Place Award.