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POTENTIAL MECHANISMS MAINTAINING A CONSPICUOUS POLYMORPHISM IN EASTERN MOSQUITOFISH (*GAMBUSIA HOLBROOKI*)

Katelyn Giltner

A THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science
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Abstract

POTENTIAL MECHANISMS MAINTAINING A CONSPICUOUS POLYMORPHISM IN EASTERN MOSQUITOFISH (*GAMBUSIA HOLBROOKI*)

Katelyn Giltner

A thesis submitted in partial fulfillment of the requirements
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Genetic variation often precedes adaptation, so the mechanisms that maintain this variation are a central topic in evolutionary biology. *Gambusia holbrooki*, or eastern mosquitofish, possesses a rare polymorphism for melanistic coloration. Melanics and typical silver males were examined for differences in behavioral and physiological traits that may impact the persistence of the melanism trait. No differences in standard metabolic rate were found via closed-chamber respirometry, but a detour task indicated that melanic males possess greater cognitive flexibility. Effects of the social environment on stress and reproductive traits were also evaluated by pairing males and comparing cortisol levels and sperm quality to individual baselines. These controlled social pairings revealed no significant relationships between social factors, stress, and reproductive traits. Future work may better illustrate the role of environmental factors on the persistence of melanism by thoroughly characterizing both competitive and reproductive interactions and evaluating potential physiological mechanisms underlying differences in cognition.

Acknowledgements

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Thanks to the state of Florida for permission to collect fishes for use in this project, and thank you to Dr. Megan Head and Dr. Meng-Han Chung at Australian National University in Canberra, who advised me on handling and collecting sperm from our *Gambusia* fishes. Finally, thank you to my friends and family for your constant support – whether in the form of dragging me out of the lab to get fresh air, listening to me blather on about my fish, or otherwise.

I truly could not have completed this project without all of your support, and I feel very lucky to have had such great help along the way. Thank you.

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Chapter 1. Metabolism and Cognition

Body coloration, either silver or melanic, was evaluated for relationships with metabolic rate and cognitive flexibility in male *Gambusia holbrooki*.

1.1 Introduction

The maintenance of diversity in natural populations is a core question in evolutionary biology since natural selection and genetic drift should favor the reduction of variation (Chesson, 2000). A few major mechanisms that contribute to variation have been identified, including heterozygote advantage (Romeo *et al*., 1989; Sellis *et al*., 2011), environmental variation over time (Lande & Shannon, 1996; Willi & Hoffmann, 2009), and frequency-dependent selection (Ayala & Campbell, 1974; Fitzpatrick *et al*., 2007). The specific processes that underlie these major categories are not as well-characterized and tend to vary between species; frequency dependent selection, for example, can be induced by predation (Greenwood & Elton, 1979), foraging strategy (Hori, 1993), or mate choice (Estévez *et al*., 2020). Given the importance of standing genetic variation in adaptation, understanding the mechanisms that maintain this variation is a key to understanding and predicting how populations may respond to change (Barrett & Schluter, 2008; Hilbish & Koehn, 1987).

1.1.1 *Gambusia holbrooki* and Male Color Polymorphism

The eastern mosquitofish, *Gambusia holbrooki* (family: Poeciliidae), is a small, live-bearing freshwater fish native to the eastern United States. Some populations of *G. holbrooki* exhibit a conspicuous polymorphism for melanistic coloration (Martin, 1977; Fig. 1.1). These melanic individuals can comprise ~1–20% of the male population, but the polymorphism is known from relatively few populations and generally occurs at a very low frequency, usually <5% of males (Horth, 2004). Across animal taxa, melanistic coloration has been correlated with a suite of behavioral and physiological characteristics due to potential pleiotropy with the melanocortin pathway (Ducrest *et al*., 2008). The relationship between melanism, physiology, and behavior have been better studied in mammals and birds (Roulin, 2004; Roulin & Ducrest, 2011). Comparatively less is known about the maintenance of this polymorphism in other taxonomic groups such as fishes.

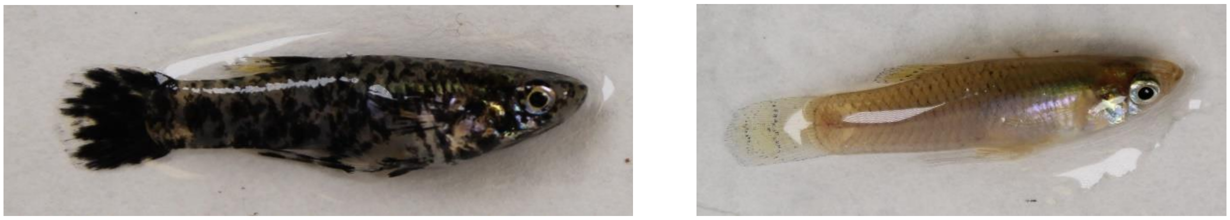

Figure 1.1 Melanic (left) and silver (right) male *G. holbrooki*.

At least one prior study in *G. holbrooki* has suggested that this Y-linked polymorphism is thermally regulated in some populations and fully constitutive in others (Horth, 2006). Other work has shown that melanic *G. holbrooki* have larger bodies, longer gonopodia (the intromittent organ used for copulation), and are more aggressive than silver males (Horth, 2003; Horth *et al*., 2010). Melanic males also tend to have more social partners than silver males in natural settings (Kraft *et al*., 2016). Female preference for male color varies; females from populations with melanic males tend to prefer them, but may also avoid copulation attempts from melanics more often (Bisazza & Pilastro, 2000; Taylor *et al*., 1996). Melanic males are captured by predators less frequently, suggesting a survival advantage (Horth, 2004). Finally, there is some evidence for frequency-dependent selection on melanism as both melanic males and females experience higher mortality rates when melanics are more common (Horth & Travis, 2002). Collectively, this evidence is equivocal, and it is not clear what mechanisms explain the maintenance of this polymorphism in male coloration. In order to identify other potential mechanisms that could be involved in the maintenance of melanism in *G. holbrooki* populations, physiological, behavioral, and reproductive traits were examined in this study to determine if they may differ between melanic and silver males and potentially contribute to fluctuating selection that maintains this polymorphism.

1.1.2 Metabolic Rate

Metabolic rate (MR) refers to the rate at which organisms uptake and expend energy and resources. MR tends to be considered a fundamental feature fueling other physiological processes, and as such, it is often called a major "pacemaker" that regulates resource use within individuals (Brown *et al*., 2004). Different measures of metabolic rate can be used to assess different activity levels and resource availability, such as basal MR (BMR; for endotherms) or standard MR (SMR; for ectotherms) as indicators of the minimum energy required for an organism to survive, or maximum MR (MMR) as an indicator of the highest energy uptake and usage that an organism can attain (Chabot *et al*., 2016; Norin & Clark, 2016).

Life history theory posits that organisms are constrained by their finite access to energy and resources – the products of their metabolism – and they must allocate these resources between self-maintenance, growth, and reproduction to maximize their likelihood of survival and their reproductive output, *i.e.*, fitness (Stearns, 1992). This physiological constraint imposed by MR thereby introduces trade-offs between traits, as allocating resources to one aspect of life history affects available resources for other aspects (White *et al*., 2022).

While direct evidence for the role of MR in inter- and intra-species life history variation is relatively limited, a handful of studies have found relationships between the two. Guppies

(*Poecilia reticulata*) display consistently higher MRs in high-predation environments, and transplants between areas with different predation levels exhibit predictable patterns of evolution in MR (Auer *et al*., 2018). Bean beetles (*Acanthoscelides obtectus*) from two different lines selected for reproduction either early or late in life display marked differences in metabolism; early beetles display higher MR early in life, and males from the early line specifically tend to mate early and frequently (Arnqvist *et al*., 2017). Stonechats from different latitudes with different migratory patterns displayed different MRs at rest even when reared in common conditions, with resident individuals having the lowest MRs and migratory individuals having the highest (Wikelski *et al*., 2003). Juvenile Atlantic salmon (*Salmo salar*) demonstrated faster progression between life stages associated with higher MR (McCarthy, 2000). Finally, a study of 138 bird species found that tropical birds tended to display lower MRs and higher survival rates compared to temperate species (Jimenez *et al*., 2014). Ultimately, MR can covary with a number of traits, ranging across mating effort, migration and activity, lifespan, and other life history traits. If MR varies between melanics and silvers, then other life history traits may likewise vary and affect the relative survival and reproductive output of melanic and silver males.

1.1.3 Cognitive Flexibility

Cognitive flexibility refers to an organism's ability to alter behavior in response to new information, and it affects an organism's ability to navigate complex environments, find mates, and seek resources (Wright *et al*., 2010). Additionally, flexibility can affect how efficiently an individual can learn from conspecific tutors (Fuss *et al*., 2021), its ability to remember previously-explored locations (Croston *et al*., 2017), and potentially to avoid predators (Vila Pouca *et al*., 2021).

Gambusia fishes tend to prefer structured habitats, typically dwelling in shallow, stagnant or slow-moving water with aquatic vegetation (Casterlin & Reynolds, 1977). Movement dynamics of *G. holbrooki* are not particularly well-characterized, but prior studies have indicated that individuals shoal in mixed-sex groups wherein a subset of individuals tend to lead group decision-making and navigation (Burns *et al*., 2012). Shoal composition varies, but groups of females tend to draw both other females seeking protection from male harassment and males seeking mating opportunities (Agrillo *et al*., 2008; Pilastro *et al*., 2003). In general, melanic males associate with more females and more conspecifics overall than their silver counterparts in both natural and laboratory populations (Kraft *et al*., 2016).

Associating with greater numbers of conspecific social partners represents an increased complexity of the social environment, which can affect cognitive flexibility (*e.g.*, in guppies, *Poecilia reticulata*; Triki *et al*., 2024). Since melanic males tend to have more social partners, they may exhibit increased cognitive flexibility that could confer benefits and aid in their persistence.

1.2 Methods

Two groups of adult *G. holbrooki* were used for the metabolic rate and cognitive flexibility trials. The first group consisted of individuals collected from Wakulla County, FL (30.211°N, -84.179°W) and the Wacissa River, FL (30.339°N, -83.992°W) in May 2019. All individuals were tested for cognitive flexibility, but only Wakulla County individuals from this collection were tested for metabolic rate. The second group consisted of individuals from Wakulla County, FL collected in July and September 2022. Husbandry and testing procedures were identical for both groups and followed approved protocols from the Institutional Animal Care and Use Committee at The University of Alabama in Huntsville (see Appendix A).

Groups of 8 individuals (N=2 melanic males, 2 silver males, 4 females) were housed in 40 L tanks containing sponge filters under a 12:12 hour light-dark cycle. Each tank was fed *ad libitum* once daily with either bloodworms or premium flake food. Tanks received a minimum 10% water change weekly.

1.2.1 Metabolic Rate

Methods for the metabolic rate assay followed (Culumber, 2016). Individuals were fasted for 24–36 hours prior to testing to ensure that they were in a post-absorptive state during measurement. Four males were measured per round of testing. To acclimate before the test, each male was placed in an opaque 500 mL Nalgene bottle containing approximately 350 mL of conditioned water and a bubbling air stone to maintain oxygen levels. One Nalgene bottle with no male inside was used and measured during each round of testing to check for consistency of background respiration rates between and throughout rounds. Bottles were placed in a 38 L water bath filled to a 10 cm depth to ensure water temperature remained consistent throughout the trial. The bath contained an Eheim-Jäger 150W aquarium heater set to 25°C and a bubbling air stone to circulate water in the bath. Males were left in the bottles for at least 16 hours (overnight).

Following acclimation, the air stones were removed from each bottle, and each bottle was flushed and filled completely with conditioned water. Once all air had been displaced from the bottle, each one was capped tightly, and one calibrated YSI ProSolo optical dissolved oxygen probe (YSI Incorporated, Yellow Springs, OH, USA) was inserted through each lid. Probes were sealed in with plumber's putty to ensure no oxygen dissolution from the surrounding air during the test. Probes recorded oxygen levels in the bottles once per second for four to five hours.

After oxygen measurement was complete, each male's blotted dry mass was measured. Males were allowed to recover for 10–20 minutes in a 3.8 L tank with an air stone and a 0.5 mL dose of API Stress Coat+ to aid in recovery. Following the recovery period, males were returned to their home tanks. Nalgene bottles were sterilized in a 10% bleach solution and rinsed thoroughly before use in the next round of testing.

To analyze variation in metabolic rate data between silver and melanic males, an analysis of covariance (ANCOVA) was performed in SPSS (IBM Corp., 2021). To estimate each male's standard metabolic rate, the first 60 minutes of measurements were discarded to avoid any effects from the initial probe insertion and sealing, and the final 60 minutes were discarded to avoid effects from hypoxic stress as oxygen concentrations decreased. A linear regression was conducted in Python (ver. 3.11.4) between the remaining dissolved oxygen measurements and time, with temperature as a covariate. The resulting regression slopes over time acted as the SMR estimates per individual.

Preliminary exploratory analyses indicated significant variation in MR across the tanks in which focal individuals were held prior to testing ($F(10,32)=14.956$, $p<0.001$). Bonferroni-corrected pairwise comparisons among tanks indicated that this effect was driven by just one of the eleven tanks used in testing as pairwise comparisons were non-significant among the remaining ten tanks ($p>0.05$). All MR measurements for the outlier tank, including background respiration rate in an empty bottle, were 1.5–2 times greater than rates measured for other tanks. This difference was likely due to either contamination or miscalibration of the probes and was unlikely to reflect a real biological difference. As such, data from that tank were discarded from subsequent analysis.

SMR data for a total of 29 individuals (N=15 melanic, 14 silver) were analyzed in an ANCOVA with a fixed factor of morph, a random effect of holding tank, and body mass as a covariate. A Shapiro-Wilk test affirmed that residuals of SMR were normally distributed.

1.2.2 Cognitive Flexibility

Methods for the cognitive flexibility assay were adapted from (Wallace *et al*., 2020). A focal male was placed in a tank filled to a 10 cm depth, wherein he needed to navigate out of a corridor, around a transparent barrier, and into a chamber containing a reward female at the opposite end of the tank (Fig. 1.2).

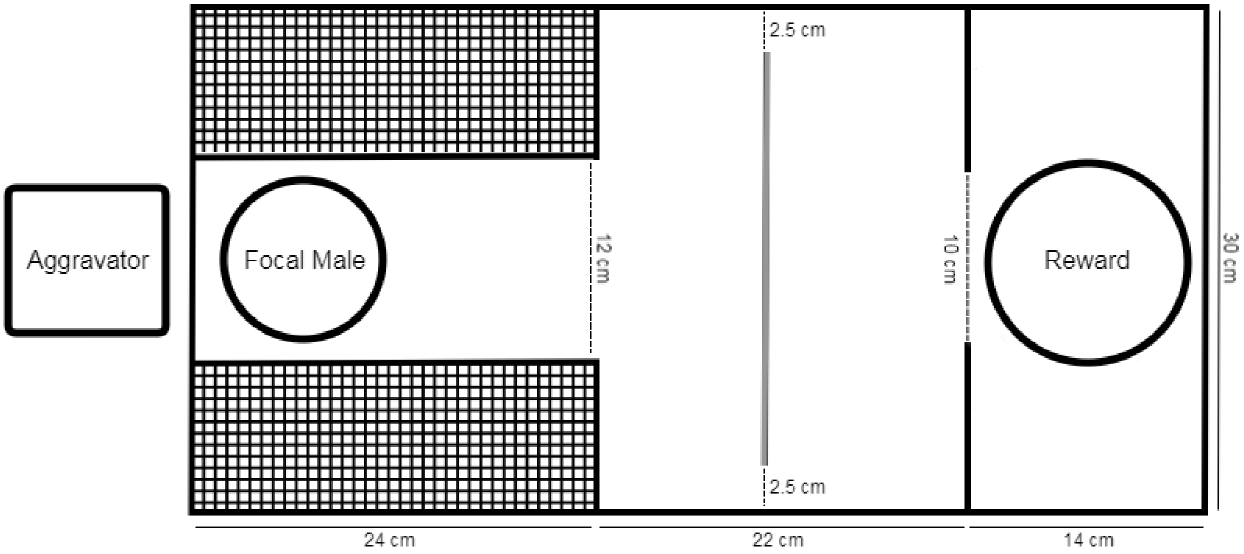

Figure 1.2 Detour arena for measuring cognitive flexibility.
The grey center line represents the clear barrier that the focal male needs to navigate around to reach the reward female.

The focal male was placed into an opaque tube at the starting end of the tank to acclimate for five minutes and minimize any effects of stress from transport. A reward female was placed in a transparent cup at the opposite end of the tank, and an aggravator male was placed in a transparent plastic cup beside the focal male’s starting point. Both the reward and aggravator were drawn randomly from different populations than the focal male to avoid any influence from previous social interactions between the individuals. All aggravators were silver males.

After the five-minute acclimation period, video recording of the trial began using a camera mounted above the tank. The opaque tube around the focal male was lifted, and he was permitted to try and navigate to the reward female for 10 minutes. Time taken to reach the transparent barrier and to enter the reward area was recorded for each male.

After 10 minutes, the focal male was removed from the tank and photographed to measure body length, then returned to his home tank. After every second trial, an approximately 20% change of the water in the test tank was conducted. Each reward and aggravator pair was used for two trials in a row, once with a silver and once with a melanic focal male, before being photographed to measure body length and returned to their home tanks. Standard length of focal males, reward females, and aggravator males were measured from photographs using ImageJ (Schindelin *et al*., 2012).

The total time taken for the focal male to reach the reward female was considered a measure of motivation, while the time between first encountering the transparent barrier to reaching the reward was used as the measure of cognitive flexibility. Individuals that failed to reach the reward female were assigned a maximum value of 600 seconds.

Analysis of cognitive flexibility data generally followed the methods described previously for metabolic rate. ANCOVA was used with the cognitive flexibility score as the dependent variable with fixed effects of male color and body size. Linear covariates included the trial start time and body sizes of aggravators and rewards since they may influence motivation. A Shapiro-Wilk test affirmed that residuals of the flexibility measurements were normally distributed. Backward elimination was used to remove nonsignificant ($p>0.1$) two-way interaction terms from the model.

1.3 Results

1.3.1 Metabolic Rate

On average, melanic males consumed $1.084*10^{-4} \pm 0.039*10^{-4}$ (±SE) milligrams of dissolved oxygen per liter of water (mg/L) each second, while silver males consumed $1.072*10^{-4}$ mg $\pm 0.044*10^{-4}$ mg/L each second (Fig. 1.3). Neither tank (F(9,17)=1.06, p=0.440) nor morph (F(1,17)=0.0284, p=0.868) significantly impacted metabolic rates. However, there was a significant effect of body mass on metabolic rate (F(1,17)=8.17, p=0.0109; Table 1.1).

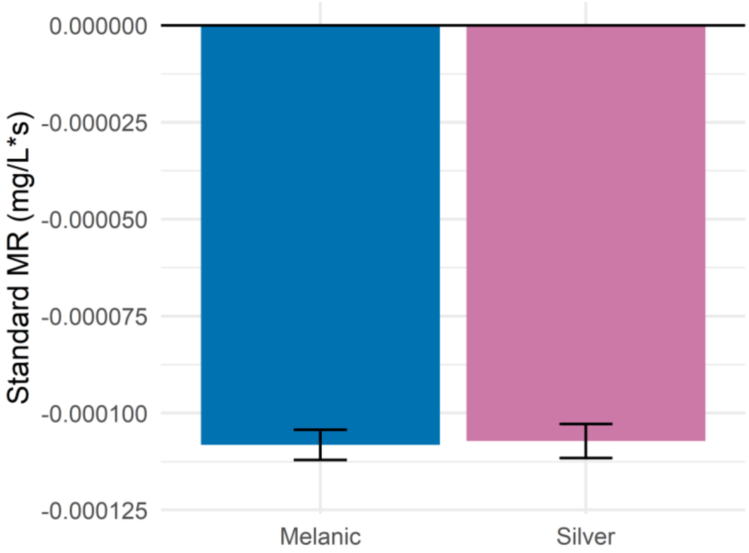

Figure 1.3 Estimated marginal means of SMR for melanic and silver males.
Error bars indicate SE. Note that the bars are negative as the SMR values indicate depletion of oxygen.

Table 1.1 ANCOVA results for metabolic rate analysis.
The main effect, Morph, indicates effect of male coloration on SMR. Home tank was included as a random effect and body mass as a covariate.

Source	F	Sig.
Morph	0.0284	0.868
Tank	1.06	0.440
Body Mass*	8.17	0.0109

* = statistically significant ($p < 0.05$)

1.3.2 Cognitive Flexibility

There was a nearly two-minute difference in cognitive flexibility between melanic and silver males (Fig 1.4). On average, melanic males took 277 ± 37 (±SE) seconds to navigate around the transparent barrier to reach a reward, while silver males took 391 ± 37 seconds. In addition to the effect of male morph on cognitive flexibility ($F(1,49)=5.20$, $p=0.0270$), there was an effect of body size of the aggravator male ($F(1,49)=8.14$, $p=0.00632$) and start time of the trial ($F(1,49)=7.01$, $p=0.0109$). Focal males tended to solve the detour task more quickly in the presence of larger aggravator males (Fig. 1.5). Although significant, the relationship between start time and flexibility was extremely weak when not accounting for morph type. Indeed, there was a marginally non-significant interaction between morph and trial start time that indicated a very strong, opposite trend for male morphs to differ in their relationship between start time and flexibility ($F(1,49)=3.961$, $p=0.052$). The two morphs had similar solution times in afternoon trials, but melanics were faster to solve in the morning becoming slower into the afternoon, whereas the opposite was true for silver males (Fig 1.6). All other covariates were not significant (Table 1.2).

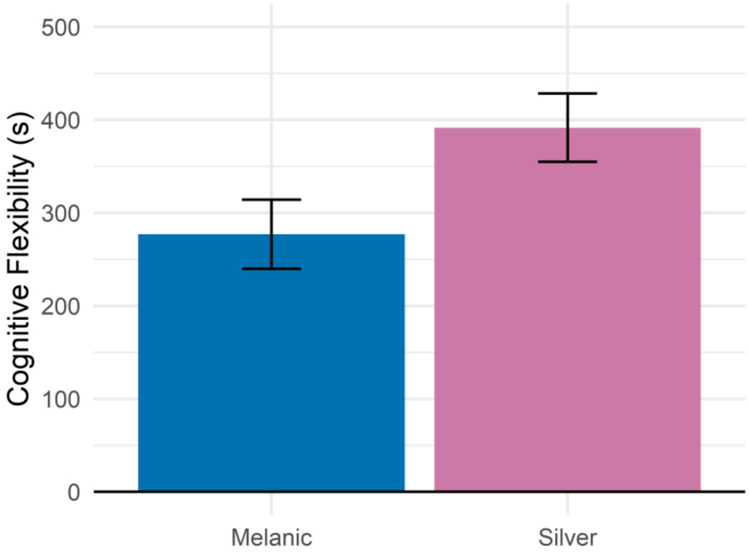

Figure 1.4 Estimated marginal means of cognitive flexibility for melanic and silver males. Error bars indicate SE.

Table 1.2 ANCOVA results for cognitive flexibility analysis.
The main effect, Morph, indicates effect of coloration (silver or melanic) on cognitive flexibility. Home tank was included as a random effect, and body length, aggravator size, reward size, and start time were included as covariates. All interactions aside from Morph × Start were removed via backward elimination.

Source	F	Sig.
Morph*	5.20	0.0270
Tank	1.15	0.335
Body Length	2.26	0.139
Aggravator Male Body Length*	8.14	0.00632
Reward Female Body Length	2.23	0.142
Trial Start Time*	7.01	0.0109
Morph × Trial Start Time	3.96	0.0522

* = statistically significant ($p < 0.05$)

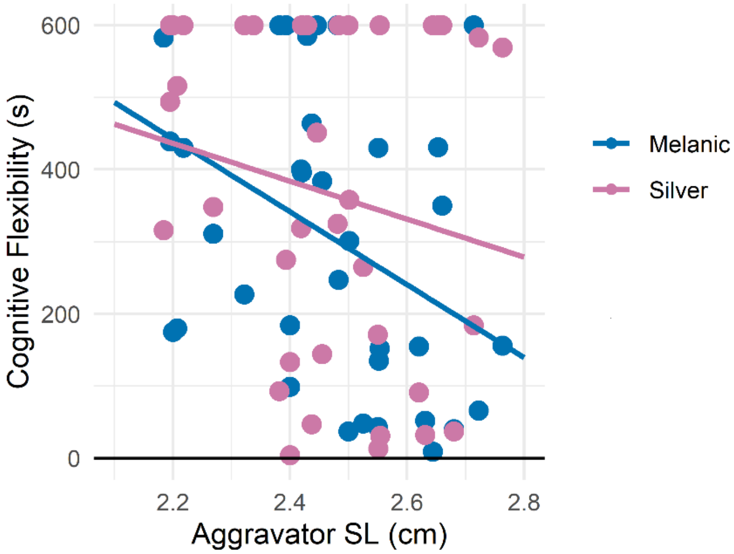

Figure 1.5 Cognitive flexibility vs. aggravator male size for melanic and silver males. Linear trendlines for each morph are included.

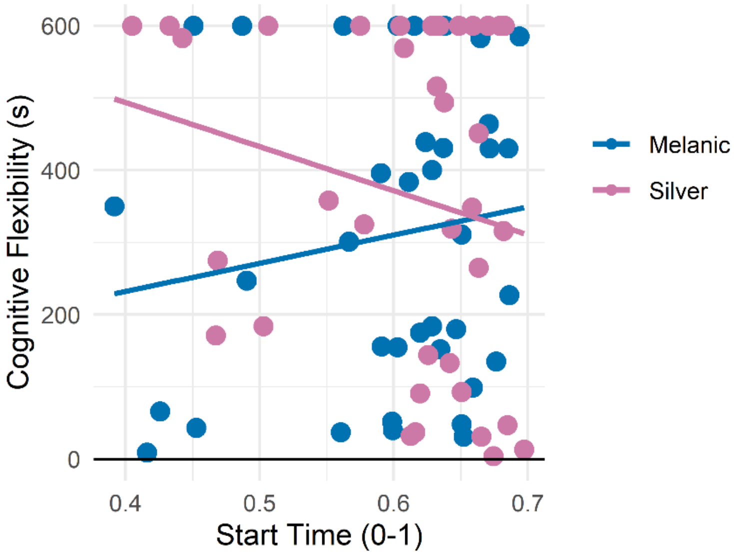

Figure 1.6 Cognitive flexibility vs. trial start time for melanic and silver males. Start time has been scaled to a 0-1 scale, wherein 0.5 equates to 12PM. Linear trendlines for each morph are included.

1.4 Discussion

Standard metabolic rates did not differ between melanic and silver male *G. holbrooki* in this study. Melanism has been found to correlate with increased metabolic rate in some systems, possibly owing to pleiotropy within the melanocortin pathway (Ducrest *et al*., 2008). However, effects of melanistic pigmentation on MR vary among other poeciliids. Within the genus *Xiphophorus*, for example, *X. helleri* displayed no differences in MR between spotted and non-spotted males (Meyer *et al*., 2006), and one study found that *X. variatus* displayed MR variation according to melanistic tailspot variation (Borowsky, 1984) while a later study of *X. variatus* found no relationship between MR and tailspot type or overall coverage of melanistic spots on the body (Culumber, 2016).

Because SMR indicates the amount of energy needed for an organism's baseline survival, it also demonstrates the cost of self-maintenance, or how much of an individual's resources are devoted just to maintaining fundamental bodily functions alone (Hulbert & Else, 2004). Higher SMR tends to correlate with a higher maximum MR, or the greatest energetic output an organism can attain, but it also suggests a higher base cost of self-maintenance, thereby reducing the basal amount of resources an individual can devote to growth and reproduction (Auer *et al*., 2017). These overhead costs may potentially attenuate any pleiotropic effects of coloration on SMR in melanic males.

Other metrics related to energy use and homeostasis may provide a better picture of differences between silver and melanic males, if any exist. In bluegill sunfish (*Lepomis macrochrirus*), SMR was not predictive of pace of life traits, but maximum MR and aerobic scope (the difference between minimum and maximum MR) were higher in bold individuals (Binder *et al*., 2016). Measuring other energetic traits like MMR or aerobic scope may provide a better picture of pace-of-life differences between the morphs that may reflect the energetic costs or benefits of being melanic or silver.

Cognitive flexibility varied significantly between melanic and silver males in this study. Melanic males displayed increased flexibility compared to silver males. The more complex social environments typically experienced by melanic males may relate to their enhanced flexibility (Kraft *et al*., 2016; Triki *et al*., 2024). This flexibility difference alone may improve the ability of melanic males to maintain larger social networks and obtain more mating opportunities, and it may also improve other traits related to cognition. Further exploration of cognitive traits and their effects on melanic and silver males would assist in determining what role this difference in flexibility might play in the persistence of melanism among populations of *G. holbrooki*.

Additionally, melanism may produce effects on eyesight as melanin holds a role of light-scattering within the retina (Price *et al*., 2008). Differences in eyesight, if they exist between the male morphs, would very likely affect success in a detour task as it requires visual evaluation of the environment, including the reward and aggravator. Examination of retinal composition or behavioral responses to visual stimuli in melanic and silver males could determine whether eyesight differences might be affecting performance during visual tasks.

The high significance of aggravator male body size on detour task performance suggests that competition between males likely has a strong impact on their dispersal. Flexibility measures decreased with increasing aggravator body size, indicating that focal males would navigate away from a larger competitor more quickly (fig.1.5). Larger males do tend to pose a greater threat of harassment to nearby individuals, which likely encourages males to move away quickly (Pilastro *et al*., 2003).

There was a significant effect of start time in the cognitive flexibility trials. Across both morphs, males performed slightly better during the morning. Although the interaction between morph and start time was marginally insignificant, there was a clear trend. Melanic males exhibited faster solution times in morning trials and slowed into the afternoon, whereas silver males exhibited slower solution times in the morning with faster solution times in the afternoon (fig. 1.6). Melanism is hypothesized to broadly affect activity, primarily due to differences in thermoregulation for dark-colored individuals, which may be especially important for ectotherms (Clusella Trullas *et al*., 2007). Though understudied, there is also evidence that pigmentation may affect diel physiological cycles in fishes (*Silurus glanis*; Valchářová *et al*., 2022). Further investigation of possible variability in activity and thermal characteristics between the morphs would aid in evaluating whether this trend in flexibility might indicate a deeper biological difference. Given that the detour task arena and surrounding environment were standardized across time, it seems unlikely that extrinsic factors (*e.g.*, lighting) were could account for this difference. Furthermore, intrinsic factors like hunger and motivation to forage could have played a role. However, if it were the case that baseline energy use was so substantial as to alter motivation to forage, we would have expected to detect differences in metabolic rate but did not.

Ultimately, this evaluation of standard metabolic rate and cognitive flexibility across melanic and silver morphs provides mixed support for differences in physiological and behavioral traits not previously tested for the *G. holbrooki* color polymorphism. SMR did not differ between silver and melanic males, suggesting that the two morphs may not vary in other associated physiological and behavioral traits. However, melanics exhibited heightened flexibility, and as such, the detour task in particular illuminated several potential avenues for future research on this polymorphism, including further study of morph-specific competitive behaviors and physiological differences. The variation in cognitive flexibility between the morphs potentially plays a role in the persistence of melanism among natural populations, as greater cognitive abilities may aid in a variety of ways such as finding a mate or avoiding predators. Further work will be necessary to determine exactly how this variation interfaces with other differences in melanic and silver male *G. holbrooki*, and to explore whether traits such as increased cognitive abilities have trade-offs in other aspects of life history.

Chapter 2. Stress and Sperm Traits

Body coloration, either silver or melanic, was evaluated for relationships with social interaction, cortisol levels, and sperm quality in male *Gambusia holbrooki*.

2.1 Introduction

Interactions between conspecifics can have significant impacts on their behavior and physiology. Social interactions between individuals can influence their physiological states over both short- and long-term scales, especially given the consistent linkage between socialization and the hypothalamic-pituitary-adrenal (HPA) axis, which controls stress and many bodily processes across vertebrate taxa (Creel *et al*., 2013). In *G. holbrooki*, competition among males for resources - especially mating opportunities - often results in a dominant, typically larger-bodied male monopolizing access to potential mates and interacting aggressively with other males (Caldwell & Caldwell, 1962; Martin, 1975).

2.1.1 Stress

Stress is a common result of any event that induces strain on an organism, and it produces suites of effects on both behavior and physiology (Øverli *et al*., 2007). Stress can be acute and occur from a brief event, or chronic and occur consistently over a prolonged period; these two types of stress differ in their physiological mediation and downstream effects (Collier *et al*., 2017). Chronic stress, for example, tends to cause a shift toward habitual behavior and reduces an organism's ability to integrate new information in behavioral decision-making (Dias-Ferreira *et al*., 2009). Social stressors have indeed been found to relate to a variety of behavioral and physiological effects; in rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*), for example, subordinate individuals displayed not only higher baseline plasma cortisol levels, but also a larger increase in cortisol when paired with a conspecific (Sloman *et al*., 2001). Trout also fail to become habituated to social stress over time, indicating that the social environment can impose chronic stress on an individual (Fernandes-de-Castilho *et al*., 2008). This heightened cortisol level also translates to reduced thermal tolerance (LeBlanc *et al*., 2011), reduced growth rate (Saulnier *et al*., 2021), increased standard metabolic rate (Sloman *et al*., 2000), and lower sensitivity to acute stressors (Jeffrey *et al*., 2014), among other effects.

Given the relationship between stress and the social environment, interactions between *G. holbrooki* could induce chronic stress. Given that melanic males are more aggressive and larger than silver males, both traits correlated with dominance in this species, the social environment may not impose stress equally between the male color phenotypes (Caldwell & Caldwell, 1962; Horth, 2003; Horth et al., 2010). If silver males tend to inhabit more subordinate social positions than their melanic counterparts, they may experience increased levels of cortisol and a myriad of downstream effects from chronically heightened stress levels.

2.1.2 Sperm Quality

Sperm quality is a trait that may be affected by stress. Sperm quality metrics, such as counts, morphologies, and motility, tend to be reduced by chronic stress, and all of these metrics can be important for fertilization success (Campbell *et al*., 1992; Kowalski & Cejko, 2019). The quality of sperm is likely to be especially important in promiscuous species, where males are also subject to sperm competition. In such species, the sperm cells of all males that mate with a given female are competing for fertilization with her limited number of eggs (Wedell *et al*., 2002).

Livebearing fishes are no exception. Research in guppies (*Poecilia reticulata*) has shown that males need more and faster sperm to attain fertilization success (Boschetto *et al*., 2011).

In *G. holbrooki*, sperm competition is highly likely, particularly when population density is high, given that multiple paternity is common among broods (Zane *et al*., 1999). The sperm qualities that affect fertilization success in *G. holbrooki* have not been well characterized, but are likely similar to factors in other members of Poeciliidae. One study found that males were more likely to successfully fertilize a female with intermediate sperm counts but did not evaluate sperm velocity or other measures of quality that likely affect success (Head *et al*., 2015). Given that stress tends to reduce sperm quality, social stressors may reduce the ability of males to successfully sire offspring (Montrose *et al*., 2008). Social stress specifically, in the form of male-male competition, has even been implicated in reduced sperm DNA integrity among zebrafish, *Danio rerio* (Silva *et al*., 2019). Since melanic males tend to hold dominant positions in social hierarchies and display aggressive behaviors toward other individuals, they may induce social stress on silver males that leads to reduced sperm quality.

2.2 Methods

This experiment was performed with adult *G. holbrooki* collected from Wakulla County, FL (30.211°N, -84.179°W) in September 2022, March 2023, and September 2023. Individuals from different collection periods were placed in separate tanks and treatment groups throughout the experiment to control for potential effects of age or acclimation to lab conditions. Each collection was housed in 40 L tanks under a 12:12 hour light-dark cycle. Tanks contained a sponge filter and a plastic plant for environmental enrichment. Each tank was fed *ad libitum* once daily with premium flake food and received a 10% water change weekly.

To facilitate pairwise size-matching and assignment to experimental groups, all males were measured for body size and gonopodium length, then sorted into four classes based on size. Males were then anaesthetized with ice water and tagged with one of two elastomer dye colors for differentiation between paired individuals during the experiment. Each male had a minimum of three weeks to recover from elastomer implantation before entering a treatment group.

Four males (one melanic and three silver) from the same size class and collection period were drawn at random from two different tanks such that male pairs during treatment would not be biased by recent interactions. Each male was photographed to later measure standard length and gonopodium length using ImageJ (Schindelin *et al*., 2012) and placed into a separate 40 L tank. These tanks were divided in half by a transparent plastic barrier with several small holes to allow for water exchange between sides. Each tank contained two unfamiliar females on the opposite side of the barrier from the male, as well as a sponge filter, a plastic plant, and two small PVC tubes for environmental enrichment. During this initial isolation phase, males were unable to see any other males to avoid inducing any potential stress from competitive interactions. Males could see (but not mate with) the females in their tank during this period to avoid effects of isolation and a perceived lack of mating opportunities on sperm traits and cortisol levels. Each male was transferred to a separate 1 L glass beaker containing 300 mL conditioned water for one hour daily during this initial isolation period. A previous study on convict cichlids (*Amatitlania nigrofasciata*) found that habituation to handling and confinement in a beaker occurred after three to four exposures, so both the isolation and treatment period lasted four days before measurements were taken (Wong *et al*., 2008).

At the end of the four-day isolation period, baseline stress levels and sperm traits were measured for each male. Water cortisol measurements approximately followed methods from

(Midttun *et al*., 2022). All conditioned water used for cortisol measurements had been allowed to age in a 100 L water reservoir for at least one week so that any cortisol present would deteriorate. Males were netted from their treatment tank to a 1 L glass beaker containing 300 mL of conditioned water. The male was left undisturbed and out of view of other fish for one hour to allow cortisol to accumulate in the water, then removed from the beaker and returned to his tank.

Cortisol was extracted from each water sample using C18 solid-phase extraction cartridges (Waters, Milford Massachusetts, USA). These cartridges were first activated with 5 mL methanol, then rinsed with 10 mL molecular-grade water. Each water sample was pumped through a cartridge at 10 mL/min using a peristaltic pump. Cartridges were then stored at -20ºC.

An ELISA kit (DetectX®, Arbor Assays, Ann Arbor Michigan, USA) was be used to measure cortisol levels from the water samples. Cartridges will be thawed at room temperature for 30 minutes, then eluted with 10 mL methanol into glass test tubes. After evaporating the methanol via bubbling at 25ºC, the remaining cortisol was resuspended into 500 uL assay buffer from the ELISA kit. The ELISA procedure followed manufacturer instructions. All samples were run in duplicates.

Sperm collection approximately followed methods from (Vega-Trejo *et al*., 2016). To strip ejaculates, each male was first anaesthetized in ice water. Then, the male was placed under a dissection scope, and the gonopodium was gently swung forward onto a glass slide coated in 1% polyvinyl alcohol (PVA) to prevent any sperm from sticking to the glass. Gentle pressure was applied to the side of the male’s abdomen using a dissection probe to eject his entire sperm reserve. The stripped ejaculate was transferred to a tube with 200 µL extender medium (207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.49 mM $MgCl_2$, 0.41 mM MgSO4, 10 mM Tris, pH 7.5).

After the procedure, males were allowed to recover separately in a cooled water bath for a minimum of 30 minutes before being returned to their home tanks.

Within 30 minutes of collection, sperm counts and velocities were recorded. Counts were recorded by first vortexing the sperm solution for one minute. Four subsamples of the sperm solution were individually applied to a hemocytometer and photographed at 100x magnification under a microscope. The photos were later used to obtain sperm counts for each subsample, and an average count was computed across all subsamples for each individual.

To measure sperm velocities, 5 µL of the sperm solution was applied to a glass slide, activated with 3 µL of an activator solution (150 mM KCl and 2 mg mL^{-1} bovine serum albumin), and covered with a cover slip before being recorded through a microscope at 400x magnification for 20 seconds. This process was repeated three times for each sample. Computer-assisted velocity measurement was performed using the OpenCASA plugin for ImageJ (Alquézar-Baeta *et al*., 2019).

Once 24 hours had passed since the initial sperm collection, each male was stripped of ejaculate again to quantify sperm replenishment rates. Procedures for this second collection were identical to the methods described above, but sperm velocities were not measured.

After being allowed to recover from the second sperm collection for a minimum of 30 minutes, the group of four males were divided into treatments. The melanic male and one silver male were placed together into one half of a 40L tank, outfitted with a barrier and tank accessories identical to the isolation tanks, alongside four unfamiliar females. The remaining two silver males were placed in another identical tank with four females.

Following the addition of all males to their treatment tanks, each tank was recorded twice daily for four days to measure social behaviors between the males. These recordings lasted five minutes each and took place in the morning and afternoon to check for variation in behaviors throughout the day. Time spent near the transparent barrier was measured and averaged across all videos for both males as a proxy for their access to females. The difference in average time spent near the barrier was computed for each pair as a proxy for dominance, or access to females. Aggressive behaviors (hereafter "attacks") incited by each individual, including chasing and biting, were also recorded.

As during the isolation period, each male was placed into a 1L beaker containing 300mL conditioned water for one hour daily to ensure continued acclimation to stressors from handling and confinement. During these habituation periods, as well as during the second cortisol measurement, both males in a pair were visible to one another.

After four days in their treatment tanks, water cortisol levels were measured according to the methods above from (Midttun *et al*., 2022). At this time, sperm were also harvested from the males and analyzed with the same methods as above from (Vega-Trejo *et al*., 2016).

Treatment effects on cortisol levels and sperm counts were analyzed with separate analyses of covariance (ANCOVA) using SPSS (IBM Corp., 2021). The differences between baseline and treatment values were computed for cortisol level and sperm count for each individual. Cortisol differences were analyzed with the fixed effect of treatment and covariates of body size, attacks, difference in time near the barrier, and size difference of the male pair. Sperm differences were analyzed similarly, but also included covariates of average female size to which the males were exposed and cortisol difference.

2.3 Results

The ANCOVAs for cortisol and sperm count differences ultimately included 17 silver males (N=12 in silver-silver pairs, 5 in melanic-silver pairs) after disqualifications due to mortality of the focal male or his partner. Analysis of cortisol levels before and after treatment indicated that the coloration of a social partner did not significantly impact stress (F(1,11)=0.0554, p=0.818). No covariates included in the model were found to be significant (Table 2.1). Likewise, treatment did not significantly affect the difference in sperm counts (F(1,9)=2.237, p=0.169), nor did any included covariates (Table 2.2).

Table 2.1 ANCOVA results for cortisol analysis.
The main effect, Partner Color, indicates the effect of a social partner's coloration (silver or melanic) on individuals' change in cortisol after socialization. Body length, negative interactions, time near the barrier, and size difference were included as covariates.

Source	F	Sig.
Partner Color	0.0554	0.818
Body Length	0.0986	0.759
Negative Interactions Received from Social Partner	0.007	0.934
Relative Time Near Barrier (focal average – partner average)	0.425	0.528
Size Difference (focal length – partner length)	0.496	0.496

Table 2.2 ANCOVA results for sperm count analysis.
The main effect, Partner Color, indicates the effect of a social partner's coloration (silver or melanic) on individuals' change in sperm count after socialization. Body length, negative interactions, time near the barrier, size difference, change in cortisol, and female size were included as covariates.

Source	F	Sig.
Partner Color	2.24	0.169
Body Length	0.236	0.639
Negative Interactions Received from Social Partner	0.320	0.586
Relative Time Near Barrier (focal average – partner average)	3.03	0.116
Size Difference (focal length – partner length)	0.133	0.724
Cortisol Difference (paired – isolated)	0.0862	0.776
Average Female Size	0.233	0.641

Additionally, descriptive statistics were computed to illustrate any general trends present in this dataset. Estimated marginal means (EMMs) of both initial cortisol and cortisol difference were similar across all treatment types (Figs. 2.1 & 2.2). The EMMs for baseline sperm counts indicated that melanic males tended to have higher amounts of sperm in isolation (Fig. 2.3) and that silvers paired with a melanic were marginally more likely to exhibit reduced sperm counts than silvers paired with another silver male (Fig. 2.4). Average sperm velocity across all males was 72.9 µm/s (Fig. 2.5), and negative interactions had no clear effect on cortisol (Fig. 2.6).

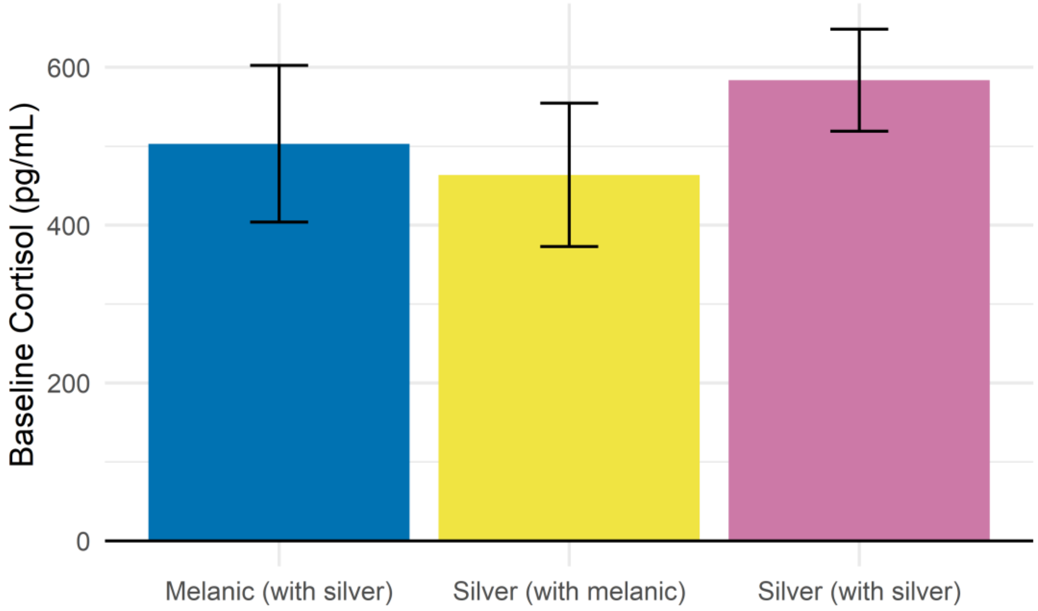

Figure 2.1 Estimated marginal means for baseline cortisol in each treatment type. Error bars indicate SE. The considerable overlap between all three error bars confirms that the baseline cortisol was not different according to morph or treatment. Baseline cortisol EMMs are corrected for body size.

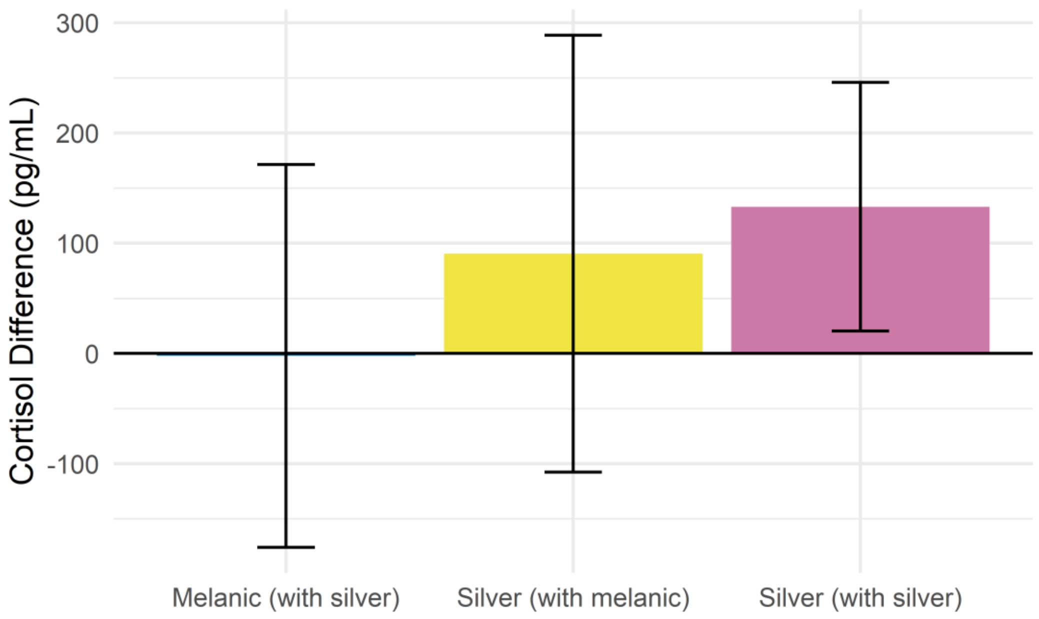

Figure 2.2 Estimated marginal means for cortisol difference in each treatment type.
Error bars indicate SE. The difference in cortisol levels before and after treatment was statistically equal across treatments. Cortisol difference EMMs are corrected for body size, size difference per male pair, negative interactions received, and relative time spent near the barrier.

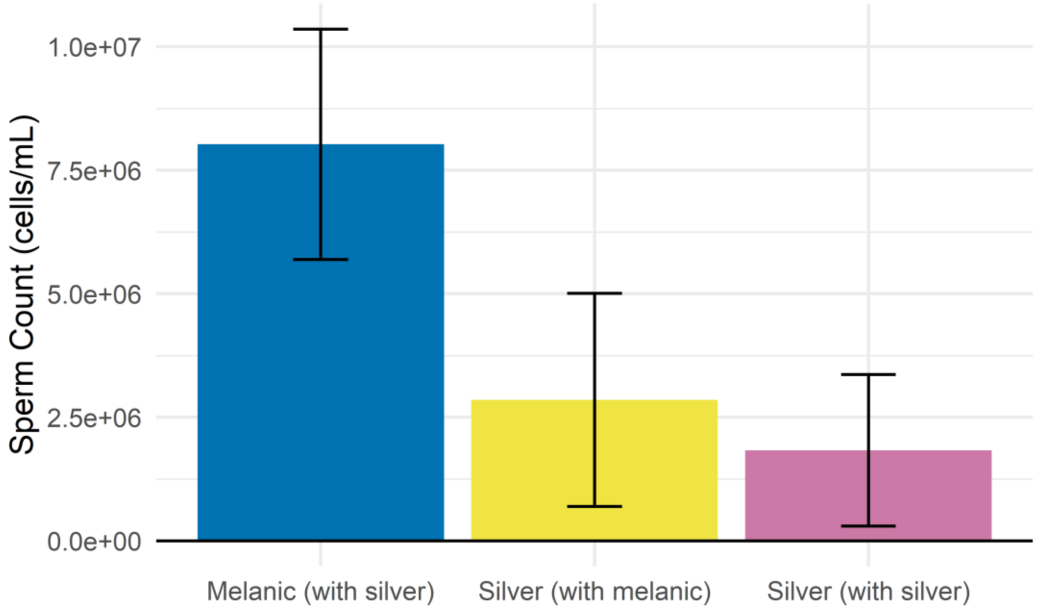

Figure 2.3 Estimated marginal means of baseline sperm counts in each treatment type.
Error bars indicate SE. Melanic males had higher initial sperm counts on average. EMMs for baseline sperm count are corrected for body length and baseline cortisol level.

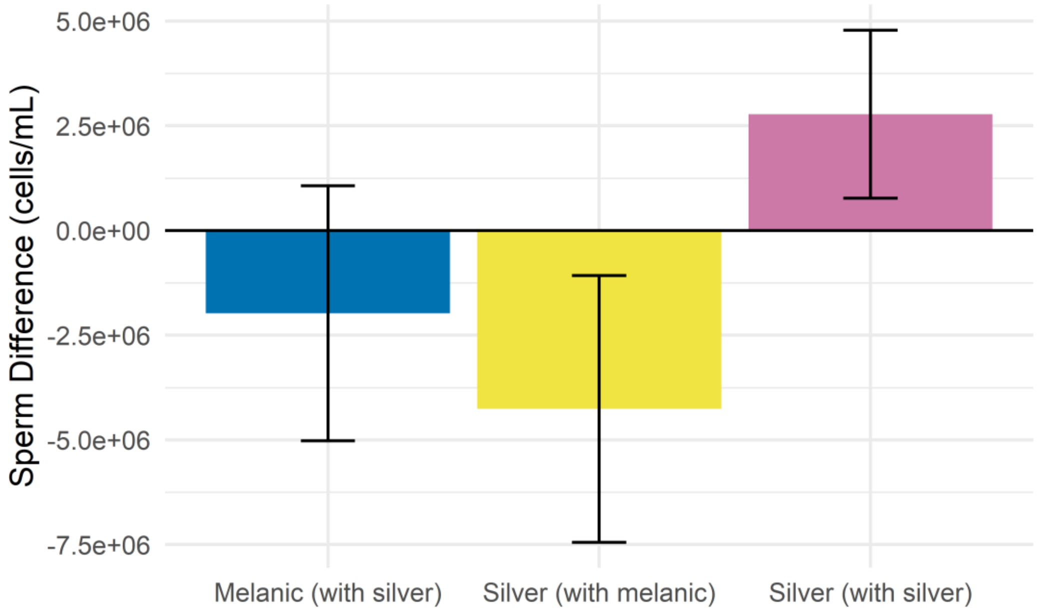

Figure 2.4 Estimated marginal means of sperm difference in each treatment type.
Error bars indicate SE. Silver males paired with melanics likely saw a reduction in sperm count, while silvers paired together likely saw an increase. EMMs for sperm count difference are corrected for body length, size difference per male pair, negative interactions received, cortisol difference, and average female size in the treatment tank.

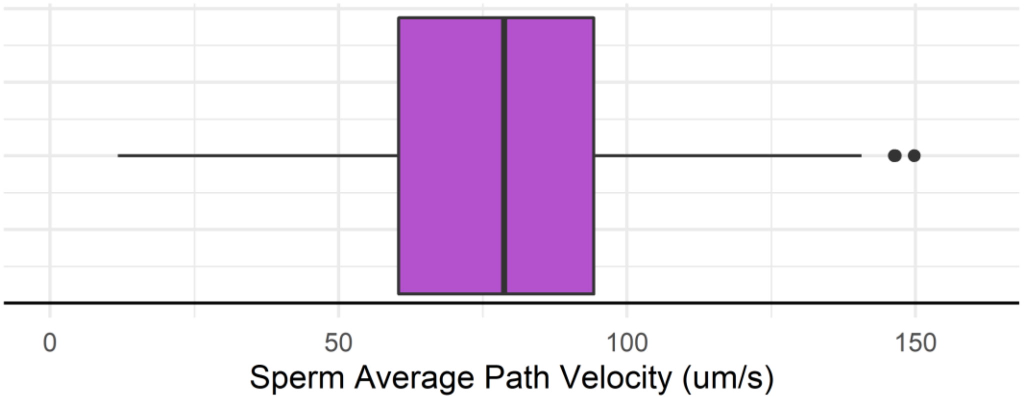

Figure 2.5 Summary of all measured sperm average path velocities (VAP).
Velocity data was included for 331 sperm cells across all three treatments and all males.

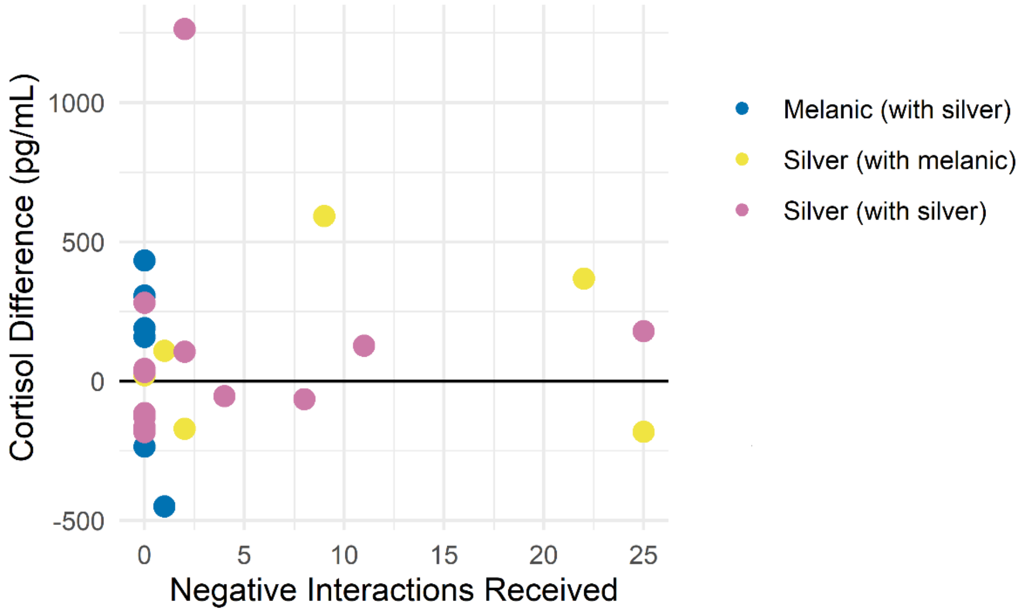

Figure 2.6 Cortisol difference vs. negative interactions received. Plot colors indicate morph and treatment.

2.4 Discussion

Neither cortisol levels nor sperm count differed between or after exposure to different social contexts. Subordinate males tend to receive more negative interactions, which included chasing and physical biting attacks, from their more dominant social partner, and these aggressive displays were expected to contribute to the chronic stress typical of a subordinate male (Fernandes-de-Castilho *et al.*, 2008). However, the number of negative interactions received across all recordings of paired behavior had no effect on cortisol levels nor a downstream effect on sperm counts. Since negative interactions served as a proxy for dominance, the lack of a relationship to cortisol could have occurred if both the dominant and subordinate individuals in a pair experience a similar increase in cortisol whether receiving or performing an aggressive display (*e.g.*, Pavlidis *et al.*, 2011). If that were the case, then cortisol levels when paired should have correlated with the combined number of negative interactions (whether received or performed), but it did not. Subordinate males may have also used submission signals, such as fleeing and hiding, to mitigate the physiological costs of aggressive engagements with a dominant male, but such behaviors were not scored in this study (Reddon *et al*., 2021). Finally, only conspicuous aggressive interactions, including bites and chases, were scored in this study; *Gambusia* also use other displays, such as fin-slapping, as hostile signals to one another, but these behaviors could not be accurately resolved in videos of paired males' behavior (Martin, 1975). More detailed records of males' behavior while paired, including time spent hiding and counting other aggressive behaviors, would provide better insights into the relationships between social interactions and physiological changes.

The difference in body sizes of each pair and relative time spent near the barrier in each treatment tank were also considered proxies for dominance. The most dominant male within a group tends to monopolize access to females, and the most dominant male tends to be the largest (Pilastro *et al*., 2003). As such, a male's time at the barrier and body size compared to his partner were considered to be additional indicators of their status within the pair. Despite the anticipated relationship between subordinance and heightened cortisol levels as mentioned above, the relative time spent near the barrier was also not predictive of cortisol difference. Again, subordinate males may be employing submission signals to mitigate effects of aggression – more detailed accounts of males' behavior while paired will provide a more thorough understanding of any hierarchical dynamics that arise.

Finally, neither the average size of visible females nor cortisol differences were significantly related to individuals' difference in sperm counts between the baseline and paired measurements. Average female size was included as a covariate due to males' common preference for larger females, but other factors like indicators of female reproductive state (*e.g.*, size of gravid spot) or female behaviors could also influence the males' sperm production (Bisazza *et al*., 1989). Cortisol differences were expected to influence sperm counts given previous work illustrating reduced sperm quality metrics under chronic stress in other fish species (Campbell *et al*., 1994), but additional analysis of cortisol-related metrics and increased sample size may be necessary to detect a relationship. Furthermore, a number of males were found to have zero or non-detectable sperm counts following their social exposure. Other researchers (M. Chung, personal communication, 21 May 2023) have observed zero sperm counts suggesting it was not an issue with methodology but may be biologically meaningful.

Obtaining an adequate sample size was a challenge to this study. Melanic males are rare in nature and therefore limiting to study. Furthermore, generating size-matched male pairs was challenging as large silver males tend to be uncommon in natural populations. A large size difference between male pairs could either introduce considerable unwanted noise during analysis or result in virtually no social interaction (*e.g.*, in *Xiphophorus variatus*, where males rarely engage larger conspecifics; Culumber & Monks, 2014). As a result, only 36 males were available for treatments. An additional 12 males were used for a pilot test to determine testing lengths and practice procedures, but could not be retested as familiarity with the testing procedures would have likely affected their responses. Further characterization of potential effects of the social environment on the male morphs would likely require additional replicates of this experiment.

The complexity of the social environment even between pairs or small groups can make it difficult to comprehensively capture social interactions, even under controlled experimental conditions. However, the effects of social interactions on reproductive investment and, ultimately, on fitness should not be ignored. Social stress can indeed affect sperm quality in fishes (Silva *et al*., 2019), and such effects may play a role in the persistence of rare traits like melanism in *G. holbrooki,* particularly given that the polymorphism itself is related to an individual's aggressive behaviors, dominance, mating opportunities, and overall experience of the social environment. Thorough examination of how conspecifics interact and careful control over experimental social groups will likely prove necessary to detect how the social environment may affect silver and melanic males differently and evaluate the impact of these differences on the persistence of the melanistic phenotype in natural populations.

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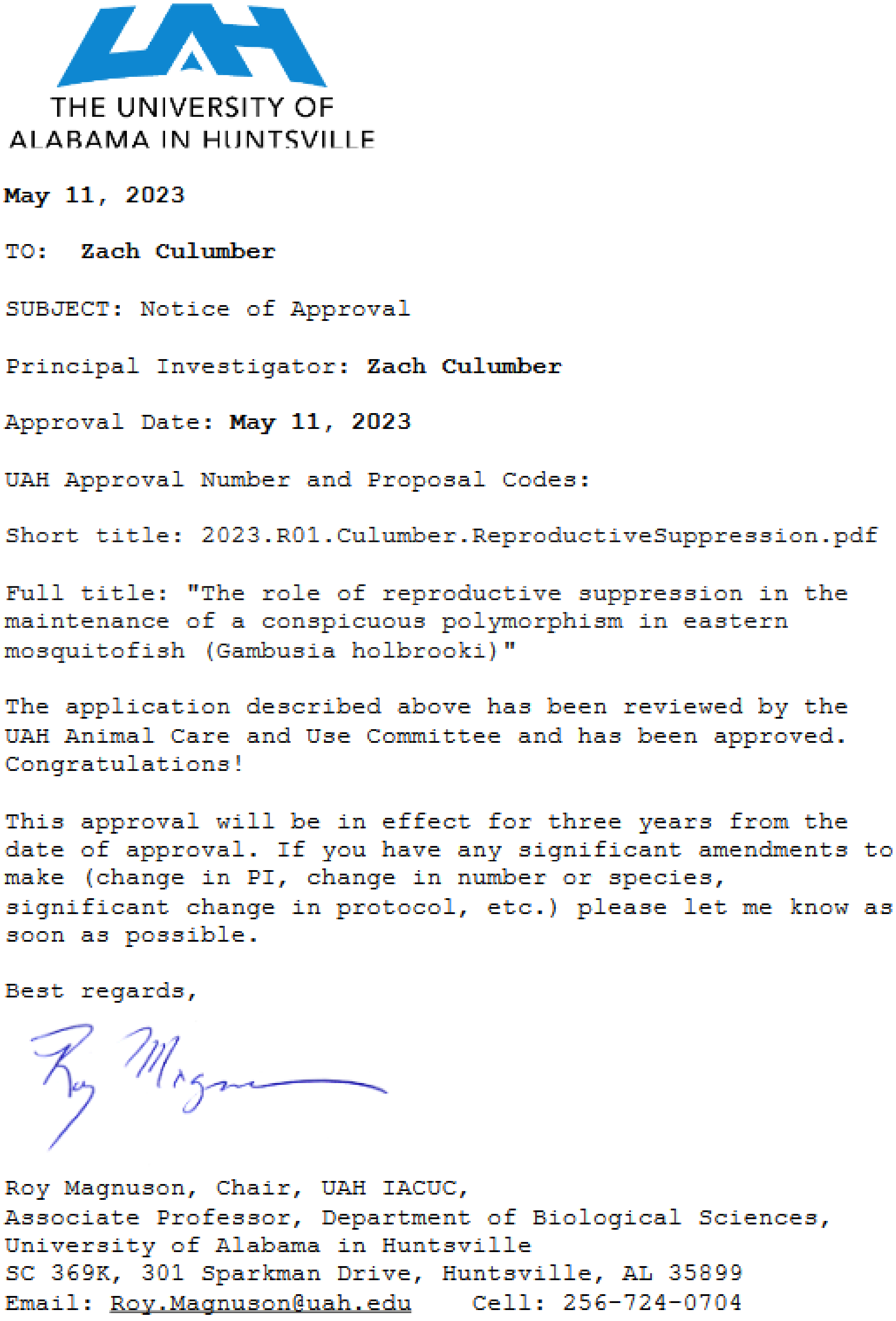
Appendix A. IACUC Permit



THE UNIVERSITY OF
ALABAMA IN HUNTSVILLE

May 11, 2023

TO: **Zach Culumber**

SUBJECT: Notice of Approval

Principal Investigator: **Zach Culumber**

Approval Date: **May 11, 2023**

UAH Approval Number and Proposal Codes:

Short title: 2023.R01.Culumber.ReproductiveSuppression.pdf

Full title: "The role of reproductive suppression in the maintenance of a conspicuous polymorphism in eastern mosquitofish (Gambusia holbrooki)"

The application described above has been reviewed by the UAH Animal Care and Use Committee and has been approved. Congratulations!

This approval will be in effect for three years from the date of approval. If you have any significant amendments to make (change in PI, change in number or species, significant change in protocol, etc.) please let me know as soon as possible.

Best regards,

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