5-2024

# Mate Choice Between Two Species of Topminnows from Dissimilar Hybrid Zones

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# Mate Choice Between Two Species of Topminnows from Dissimilar Hybrid Zones

by

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A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of Honors Requirements

May 2024

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Joyce Inman Ph.D., Dean
Honors College

# ABSTRACT

Understanding the origins of reproductive isolation between closely related species is foundational to evolutionary and ecological research. Recent reviews have documented contemporary hybridization between a large portion of species. A greater knowledge of reproductive isolation and its components are vital to expanding conservation efforts for threats such as those to species persistence and community processes. However, there is a lack of clear understanding of how isolating mechanisms evolve throughout many systems where hybridization is prominent. Individuals within the *Fundulus notatus* species complex (*F. notatus, F. olivaceus,* and *F. euryzonus*) readily hybridize at many contact zones replicated throughout their broadly overlapping ranges in the southeastern United States. However, recent work has highlighted high variability in hybridization rates across replicated zones (zones range from <1% to >30% individuals of hybrid ancestry). To better understand the mechanisms controlling hybridization rates across species contact zones, I conducted mate-choice trials between individuals from populations within the *Fundulus notatus* species complex that freely hybridize and those that do not. The experimental set up allowed individuals free choice of a conspecific or heterospecific over the course of 6-hour trials. During that time, movement was monitored and tracked for each individual through an artificial intelligence software integrated into video cameras. From there, I used temporal proximity of individuals as a metric of mate preference and to quantify the mode of sexual selection. I observed significant species and population differences in mate preferences. Female *F. notatus* exhibited greater selectivity compared to *F. olivaceus.* Within *F. notatus*, there were different levels of species recognition among the two

populations (Ouachita population and Pascagoula population). Additionally, body size influenced mate choice differently between the species. My results allow us to consider sexual selection as the driver of reproductive isolation among the *F. notatus* species complex. Future research on the genetic basis of species recognition and mate choice behaviors offers promising opportunity to deepen the understanding of reproductive isolation between closely related species.

Keywords: Reproductive isolation, hybridization, *Fundulus notatus* species complex, mate choice trials

# DEDICATION

I would like to dedicate this to my parents, Micheal and Edith Davis, who have shown me unconditional love and support throughout my academic journey at the University of Southern Mississippi.

# ACKNOWLEDGMENTS

I am deeply grateful to Dr. Schaefer for his exceptional guidance and patience throughout this process. I would also like to thank Emilee Holderness for her support in both trial running and experimental design. Lastly, I thank the Honors College for allowing me to grow professionally and academically throughout the entirety of my college career.

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# LIST OF ILLUSTRATIONS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of Variance |
| FIS | Inbreeding Coefficient |
| USM | The University of Southern Mississippi |

# CHAPTER 1: INTRODUCTION

Reproductive isolation serves as a fundamental concept in understanding speciation and plays a role in defining what constitutes a species. According to the widely accepted Biological Species Concept, a species is characterized as a group of interbreeding natural populations that are reproductively isolated from other groups (Coyne, 2004). Reproductive isolation between species is maintained by various isolation mechanisms, which can be classified as prezygotic or postzygotic barriers. Prezygotic barriers manifest before the production of offspring and include behavioral and morphological differences, including mate preferences (Duvernell et. al., 2007). These barriers prevent mating or hinder successful fertilization between individuals of different species, reinforcing reproductive isolation. Postzygotic barriers occur after the formation of the zygote and include genetic incompatibility and reduced hatching success (Viguera et al., 2008). These barriers hinder development and survival of hybrid offspring. Hybridization, the interbreeding between individuals from different species, only occurs when reproductive isolation is incomplete. This phenomenon highlights the dynamic nature of reproductive barriers and illustrates the importance of understanding the mechanisms behind reproductive isolation in the context of speciation.

Exploring animal mate choice and its role in prezygotic isolation is crucial in evolutionary biology. As a fundamental component of sexual selection, mate choice deeply affects various evolutionary processes, particularly speciation (Servedio, 2007). By choosing mates based on specific traits, animals play a part in the divergence of populations and the development of unique genetic identities within different species (Lindsay et al., 2019). Mate preference doesn't only influence how species evolve and

differentiate; it also plays a significant role in how species come together through hybridization (Rosenthal, 2013). When individuals from different populations or species interact, their preferences in mates can determine both the possibility and the nature of hybridization. This has a profound effect on the evolutionary paths of populations and the preservation of genetic diversity within communities (Duvernell et al., 2004) Hence, to fully grasp how evolution and species diversification work, it's essential to study how prezygotic barriers, specifically mate preferences, affect hybridization.

Research consensus suggests that the sex which invests more energy in reproduction tends to become the more discriminating one when it comes to selecting a mate (Darwin, 1859; Lehtonen et al., 2016). Generally, in the animal kingdom, females invest more in producing eggs than males do in producing sperm (Schlupp, 2018). This imbalance in reproductive investment sets up Darwin's seminal theory of sexual selection, which is divided into two main components: competition within the same sex, usually among males, for access to mates, and selective choice of mates, a process often driven by females (Darwin, 1859). Although historical perspectives depicted females as passive in mate choice, contemporary research reveals instances of role reversals of both sexes engaging in both competition and choice (Amundsen & Forsgren, 2001; Servedio, 2007). The evolving landscape of mating systems extends beyond traditional binary frameworks, reflecting the diversity inherent in modern evolutionary biology. In a broader context, this helps us understand the evolutionary process and factors driving biodiversity.

Experimental design has significantly influenced the measurement of mate choice, influencing outcomes and insights into selection mechanisms. Variations in design,

particularly in the number of options presented to subjects during trials, can significantly affect the assessment of mate preferences. Tests can be categorized as choice or no-choice experiments (Wagner, 1998). Wagner (1998) describes standard methods utilized in animal behavior research to assess female preferences, particularly in birds, amphibians, and fish. In amphibians, the two-speaker choice test is common, where a receptive female is positioned between two speakers emitting synthetic calls differing for each potential mate. When the female approaches one of the speakers, her choice indicates a preference based on the call's variation. For birds, arena tests are common, with females placed in an environment containing live males with differing traits, or speakers broadcasting different song types. A common choice test for fish involves setting up an arena divided into three or more sections, with the end sections separated from the center section by glass or plexiglass barriers (Wagner, 1998). The two ends house different males, each displaying distinctive traits, while the center section is for a female, initially placed in a removable compartment. Following an acclimation period, the barrier surrounding the female is removed. Researchers then record the duration the female spends in proximity to each male or engaging in courtship behaviors. If females exhibit a significant inclination toward one male, it suggests a preference for the traits associated with that male (Wagner, 1998). This choice method does not involve interaction and spawning but allows researchers to assess female preference for specific male traits. Conversely, in a no-choice test, a single potential mate is presented to the focal individual, usually a female, with actual spawning recorded. This setup aims to study the female's reaction to a mate with predetermined traits without offering her alternatives. The mate is introduced into the arena, which also contains appropriate

spawning substrate to encourage natural reproductive behaviors. Following an acclimatization period, researchers track the number of eggs produced over a set timeframe. This method allows for the assessment of mate preference through the quantity of eggs spawned (Rundle & Schluter, 1998). Exploring how experimental design influences mate choice measurements, Dougherty (2015) conducted a meta-analysis comparing the outcomes of choice versus no-choice setups within specific species, traits, and sex groups. The analysis revealed that no particular design consistently yields more accurate insights into mating preferences. Interestingly, significant variations in preference strength and the intensity of sexual selection were documented across different experimental setups (Dougherty, 2014). This highlights the crucial impact of experimental design on understanding mate preferences and sexual selection mechanisms.

Another experiment, performed by St. John & Fuller (2019), highlights the importance of assessing the costs and benefits across different experimental setups when studying fish behavior. They conducted comparisons among three different assays and three different metrics to measure mate preference, emphasizing the necessity of considering multiple approaches (St. John & Fuller 2019). Dichotomous choice tests offer insights into female preference but may oversimplify social dynamics present in mate selection. These tests often neglect the natural interactions and competition among male fish, leading to a lack of realism in the experimental set up. Additionally, the data collection process for dichotomous choice tests can be tedious and may not fully capture the nuances of mate preference. On the other hand, non-choice assays provide means to quantify reproductive output, offering a different perspective on mate preference.

However, these assays may overlook important aspects of courtship behaviors and competition among males, leading to a reduction in realism. Although non-choice assays may be easier to execute, their simplicity comes with the cost of excluding vital components of natural mate selection (St. John & Fuller, 2019). Ultimately, researchers must carefully weigh the trade-offs between experimental realism and ease of execution when designing studies on fish mate preference. Integrating elements of social dynamics and competition into experimental setups, while maintaining feasible data collection methods, can lead to a more comprehensive understanding of mate preference (St. John & Fuller 2019).

The *Fundulus notatus* species complex presents an intriguing system for studying the relative significance of various factors that contribute to reproductive isolation between species, as well as the ecological determinants shaping species distribution (Blanchard, 1996). Two species within this complex, *Fundulus notatus* and *Fundulus olivaceus*, offer promising opportunities for investigating the mechanisms underlying speciation in natural populations. The blackstripe topminnow (*F. notatus*) is found in the Gulf Coastal Plain, ranging from central Texas to Alabama, extending northward into formerly glaciated regions of southern Michigan and Wisconsin, and westward as far as central Kansas (Thomerson, 1966). The blackspotted topminnow (*F. olivaceus*) has a distribution that broadly overlaps with *F. notatus* (Fig 1), spanning from east Texas to Florida, but only reaching as far north as the Shawnee Hills of southern Illinois and the Ozark Highlands of southern Missouri (Thomerson, 1966). Morphologically, the two species are similar throughout their ranges (Braasch & Smith, 1965; Thomerson, 1966). However, a single distinguishing characteristic, the presence or absence of distinct,

regular, dark black dorsolateral spots, typically distinguishes the two species (Thomerson, 1966). The mating system observed in the *Fundulus* genus includes spawning daily, with females depositing up to eight eggs within vegetation. Fertilization takes place externally, by females depositing eggs into the surrounding water. By depositing their eggs in specific locations, females are effectively choosing mates that inhabit those areas.

Figure 1

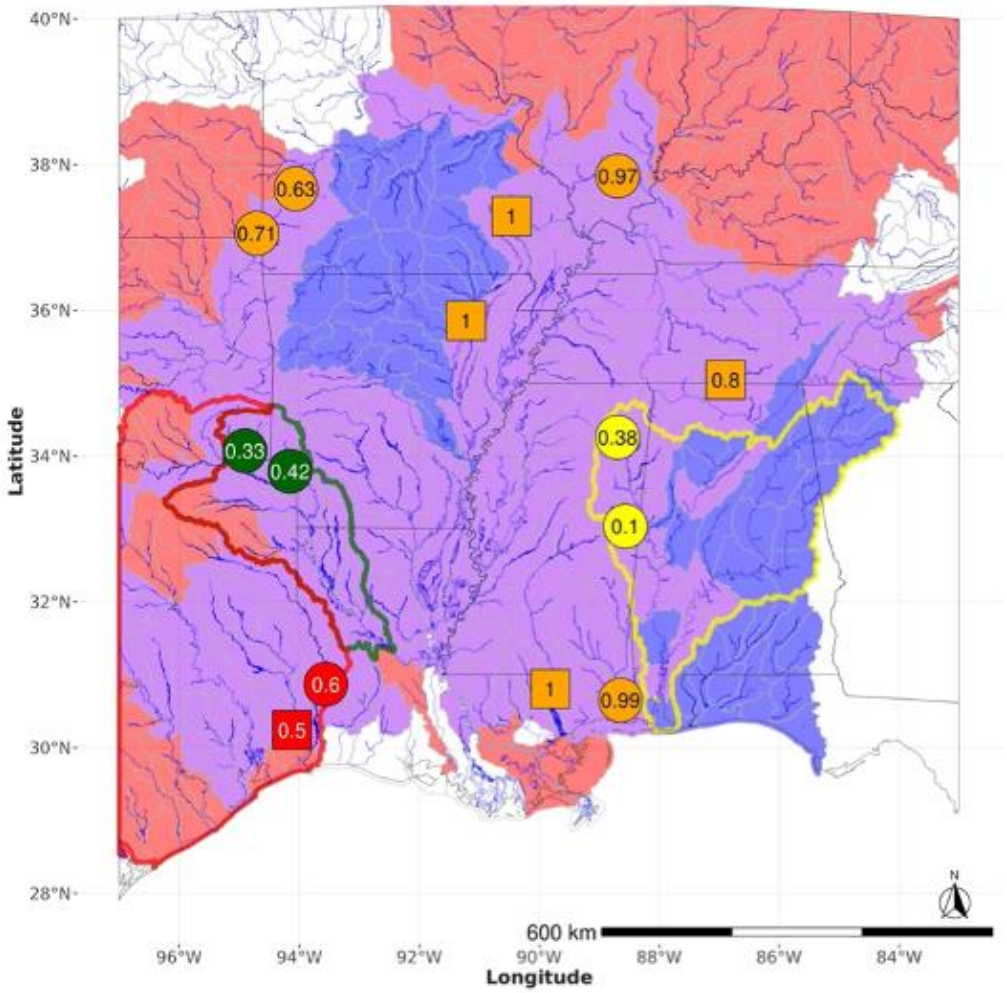


*Figure 1: Map of species distributions and contact zones in Southeastern United States. FIS values included in geographic landmarks designating contact zones. Red shading is* F. notatus *range. Blue shading is* F. olivaceus *range. Purple shading is Species overlap. Red landmarks are Western Gulf Slope. Green landmarks are Ouachita River. Yellow landmarks are Mobile River Basin. Orange landmarks are Coastal & Mississippi Basin.*

Contact zones between these species are common within their overlapping ranges, offering opportunities for potential hybridization. Nevertheless, varying degrees of hybridization are observed in these contact zones across the southeastern United States. In these contact zones, *F. notatus* have been observed to be more divergent than *F. olivaceus* (Duvernell et al., 2007). Research conducted on mate selection (likelihood of spawning in no choice trials) and the viability of backcross hybrid offspring (success of hatching) revealed indications of both prezygotic (conspecific mate preference) and postzygotic (low hatching success) barriers (Vigueira et al., 2008). It has been shown that ecological gradients within contact zones influence hybridization rates (Duvernell & Schaefer, 2014; Schaefer et al., 2016). Factors such as habitat homogenization, endocrine-disrupting compounds, or reduced environmental mediation of reproductive isolation mechanisms may also affect hybridization rates (Duvernell & Schaefer, 2014). Duvernell et al. (2014) conducted a study to explore phylogeographic effects on hybridization, sampling nine contact zones between *F. olivaceus* and *F. notatus* throughout their geographic range. A noteworthy observation in their study was the phylogeographic trends in hybridization rates exhibited across isolated drainages. The contact zones in the Mississippi River Basin (including the Pascagoula population) had low hybridization, virtually absent in some zones (Duvernell & Schaefer, 2014). In the Red River Basin (including the Ouchatia population) and the Mobile Basin, a higher

prevalence of hybrid classes was observed. They noted variation in hybridization dynamics among drainages, ranging from nearly random mating to a complete absence of hybridization (Duvernell et al., 2023). These contact zones paint a complex picture of how these species interact and how their genetic isolation has been maintained over time.

To investigate the influence of prezygotic isolation between populations with varying tendencies towards hybridization, I conducted experiments aimed at examining mate choices among individuals from these groups. Specifically, my focus populations were characterized by either high or low hybridization rates. My study looked at individuals choosing between mates from their own species (conspecific) or from another species (heterospecific), from the Ouachita population, known for its higher hybridization rates, and the Pascagoula population, where hybridization occurs less frequently (Duvernell et al., 2023). The core of my investigation rested on the belief that how I set up the experiment plays a crucial role in accurately determining mate preferences. To this end, I utilized free-choice arenas for my mate-choice trials, allowing individuals to freely choose their mates based on natural preference. I also measured how often and how long associations happened, in effort to determine a detailed look at mate choice behavior.

My hypotheses encompass several aspects. Firstly, I aim to affirm that *F. notatus* demonstrates greater selectivity in mate choice compared to *F. olivaceus*. If *F. notatus* has stronger mate choice, I expect to observe lower association distance between conspecific individuals. Association distance refers to the special proximity between individuals of a species within the experimental set up. In the context of mate choice, a lower association distance indicates that individuals are more selective in their choice of mates, as they associate with specific conspecific individuals over others. Secondly, my

objective was to investigate whether the reduced discrimination in mate selection among *F. notatus* individuals from the Ouachita population, compared to those from the Pascagoula population, was attributed to prezygotic isolation mechanisms. Although higher hybridization has been observed in the Ouachita population, the precise factors driving this remain ambiguous (Duvernell et al., 2023). I expect to observe lower association distances between conspecific individuals in the Pascagoula population. Lastly, I hypothesized that males of both *F. olivaceus* and *F. notatus* would exhibit lower distances to the spawning media compared to the females. This research sought to further the understanding of the evolutionary implications of discrepancies in hybridization among contact zones and present an experimental set up that provides consistent and repeatable results for studying mate-choice.

# METHODS

*Fundulus notatus* and *F. olivaceus* were sourced from parental populations residing in two distinct regions: the Pascagoula (Black Creek and Pascagoula River near Hattiesburg, MS) and the upper Red River (Twelvemile Bayou near Shreveport, LA) drainages. Prior research has indicated that hybridization rates in the Pascagoula region are minimal, with less than 3% of individuals displaying hybrid ancestry (Duvernell et al., 2023). In contrast, the contact zone near Twelvemile Bayou has been identified as an area with extensive hybridization among *F. notatus* and *F. olivaceus* populations (Duvernell et al., 2023). The brood stock for the trials was carefully selected from areas outside of recognized hybrid zones to ensure the integrity of the experimental populations.

Dipnets were used to sample a total of at least 20 adults that were transported to mesocosms at the Lake Thoreau Environmental Center. These individuals served as the brood stock that spawned the eggs that were used in the trials. This approach was done to avoid potential bias stemming from learned behaviors. The eggs that hatched into offspring were held in the mesocosms until trials commenced. Each trial involved the random selection of one male and one female from each species, with trials differing in the source population, either high or low in hybridization. Fish were naïve when subjected and only put through one trial. Prior to each trial, all fish were anaesthetized with MS-222 to facilitate temporary immobilization and then marked on their dorsal surface using elastomer tags (Northwest Marine Technology). Female *F. notatus* were marked with yellow, and males with orange, whereas females of *F. olivaceus* were marked with pink, and males with red. The fish were then transported to the USM Wet

Laboratory and acclimated in the arena where the trials would take place with a 24-hour adjustment period and minimal human interaction.

The assessment of association distance took place in an arena measuring 3.6 m long, 0.6 m wide, and 15 cm deep, featuring a gravel substrate and two yarn spawning mops placed at 1.8 m apart. A unique aspect of my approach was the allowance for all individuals to move freely, with association distances assessed at regular intervals (Fig. 2). Five cameras positioned above the arena captured non-overlapping sections, taking pictures every 30 seconds for a duration of 6 hours. TensorFlow AI software was employed to detect fish in the images, record their x and y coordinates in the arena, and identify the color of their elastomer marks. Data was only retained in 30 second intervals when all four individuals were recorded, and all six measurements (Fig 2) could be made.

Figure 2

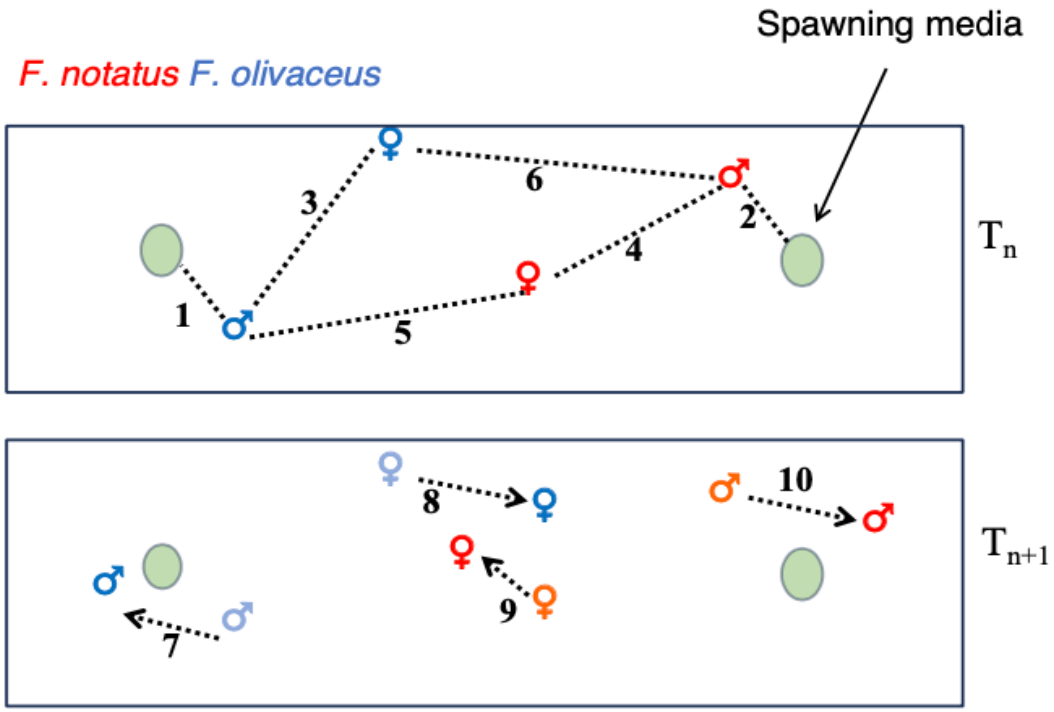



*Figure 2. Digital render of experimental set up and quantification of association distance measurements.*

*1-2 male distance to. Spawning media*
*3-4 conspecific distance*
*5-6 heterospecific distance*
*7-10 Individual Movement from $T_n$-$T_{n+1}$*

Statistical analysis, conducted using R version 4.2 (R Core Team, 2021), included testing for differences in association distance (the difference between square root transformed heterospecific and conspecific) between species and populations (maximum likelihood and ANOVA) using nested mixed models (lme4 package) with trial as a random effect. The fixed predictors were species, conspecific, population, and the size ratio categories. Additionally, I tested for species and population differences in distance from spawning mops.

# CHAPTER 3: RESULTS

## Overview

A total of 14 trials were conducted over the course of the experiment. Each trial involved introducing a male and a female of each species into the tank, where they were allowed to roam freely and interact. The AI system successfully detected and identified 80% of the fish present in the tank across all trials (e.g. in a typical 60 minute period, there would be 384 fish identified out of the maximum 480 possible). For each detection the following data was included: the probability that the object detected was a fish, the most likely elastomer color, the probability of that color being correct, the time, which camera it was recorded on, and the coordinates within the shot where the fish was located. The AI system occasionally made errors in color detection (misidentification of one color mark for another), with an average false rate of less than 1% per trial. All AI data was reviewed, and corrections were made prior to analyses.

## Conspecific Association Distance

My results revealed a significant interaction between conspecific association distance and body size, indicating distinct patterns within the females of each species (species: $X^2$=46.7, P≤0.001, species: size: $X^2$=6.3, P≤0.012). Specifically, female *F. notatus* exhibited a preference for smaller males, whereas female *F. olivaceus* displayed a preference for larger males. To quantify these preferences, a ratio of female to male size for each trial was calculated. Based on these ratios, 3 roughly equal sized groups were created (Fig. 3).

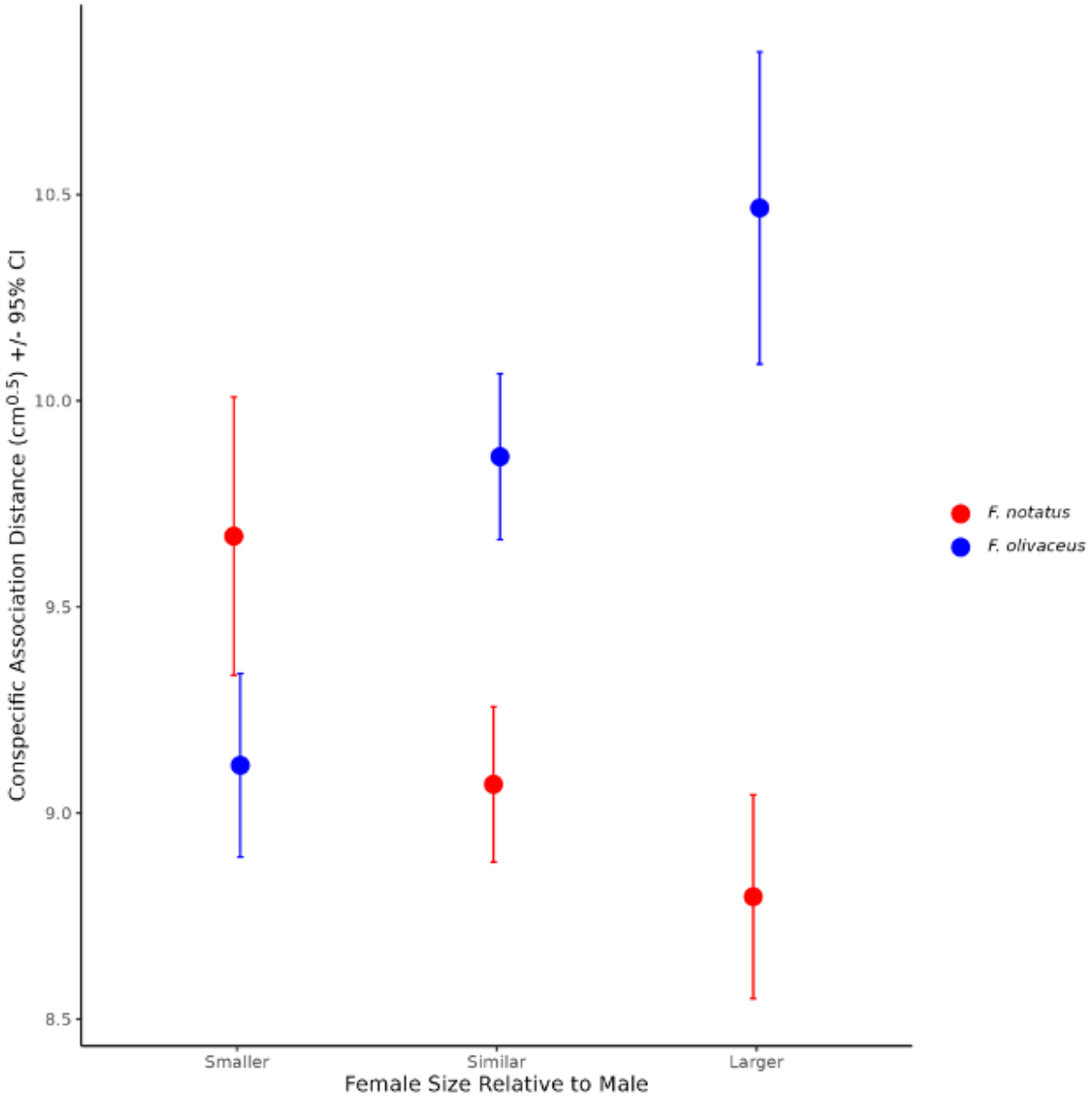



*Figure 3: Mixed-effect graph comparing F. notatus (red) and F. olivaceus (blue) distance to male conspecifics of a certain body size relative to female body size. The x-axis in the plot represents the trials categorized on the calculated ratios of female to male size.*

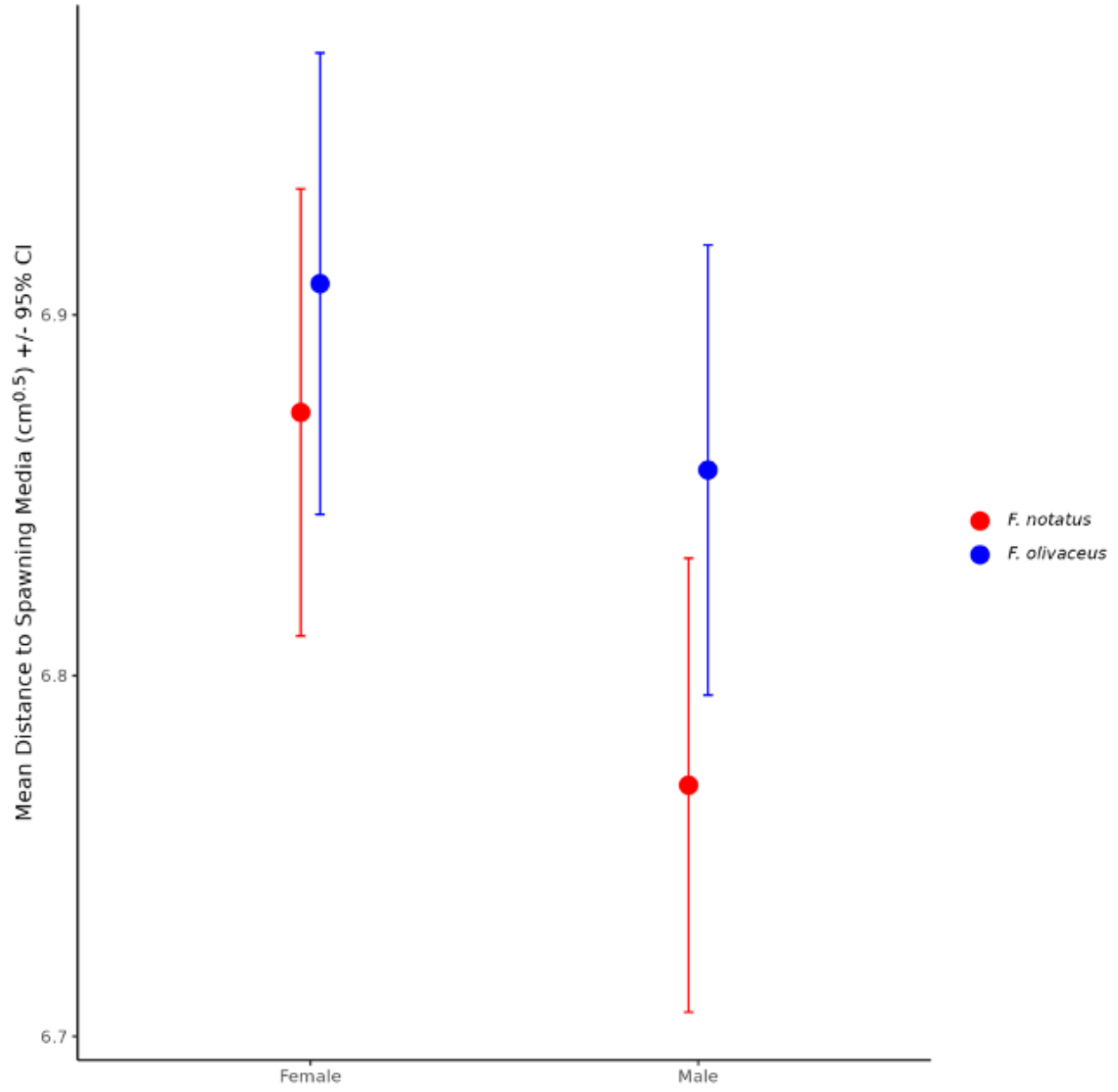
**Distance to Spawning Media**

My analysis revealed that male individuals displayed a tendency to be in closer proximity to the spawning media when contrasted with their female counterparts.

($X^2$=6.6, P<0.04). There was no significant difference is distance in distance from spawning media between *F. notatus* and *F. olivaceus* (Fig. 4).

*Figure 4: Mixed-effect graph comparing F. notatus (red) and F. olivaceus (blue) distance of males and females to the spawning media.*

**Association Distance by Clade**




My analysis revealed females of *F. notatus* exhibited a significantly greater proximity to conspecific males compared to females of *F. olivaceus* ($X^2$=46.7, P<0.001; Fig. 5). Furthermore, I observed a significant interaction between species discrimination and population with *F. notatus* ($X^2$=37.9, P<0.001; Fig. 5). Specifically, populations of *F.*

*notatus* exhibited greater heterospecific distances compared to conspecific distances, in contrast to *F. olivaceus* populations. Although not statistically significant, patterns within *F. notatus* populations mirrored field observations of hybridization rates, with greater species discrimination observed in the Pascagoula (minimal observed hybridization) and Ouachita populations (extensive hybridization).

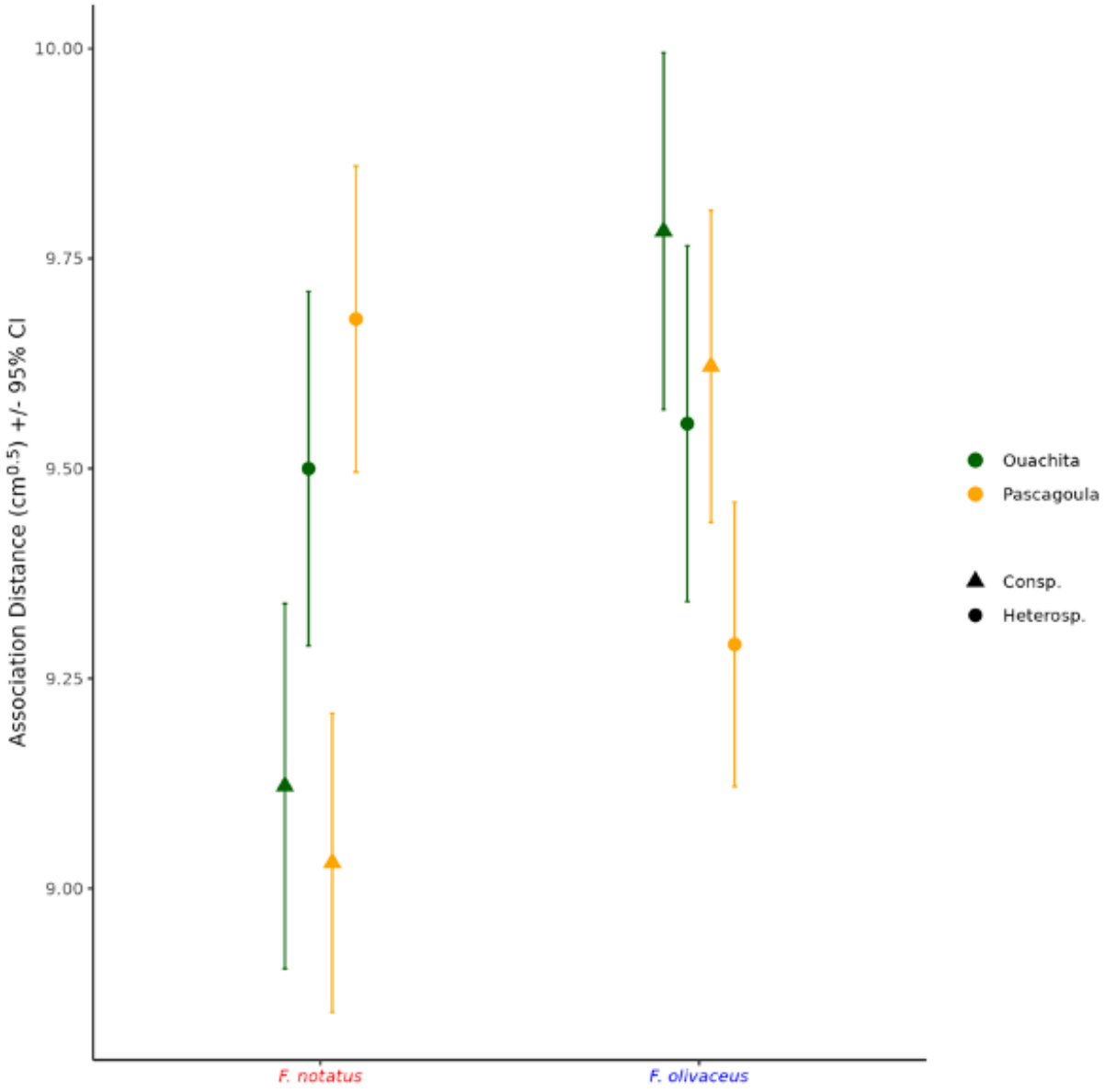



*Figure 5: Mixed- effect graph comparing F. notatus and F. olivaceus sourced from Ouachita (green) and Pascagoula (orange) populations association distance to conspecifics and heterospecifics.*

## CHAPTER 4: DISCUSSION

My study's finding provide support for each of my three hypotheses. Firstly, the hypothesis proposing greater discrimination in *F. notatus* compared to *F. olivaceus* was supported by the observed greater difference between heterospecific and conspecific association distances in *F. notatus*. Secondly, although the data regarding discrimination in *F. notatus* populations did not reach statistical significance, there was a suggestive trend implying lower discrimination in the Ouachita population, aligning with the second hypothesis. Lastly, the hypothesis predicting closer proximity of males to the spawning media is validated by the observed distances, indicating a clear association between male fish and the spawning media.

My findings provide insights into female mate choice behaviors within closely related species of the *Fundulus notatus* species complex. My data suggest that female choosiness within a choice arena varies significantly between species and among clades. I find significant disparities in species discrimination between the populations, as evidenced by conspecific and heterospecific association differences. Specifically, *F. notatus* populations exhibited greater heterospecific than conspecific distance compared to *F. olivaceus* populations, in support of the hypothesis that *F. notatus* exhibits greater discrimination than *F. olivaceous.* This finding also supports the idea that mate choice in *F. notatus* females is likely the driver of hybridization rates. Furthermore, I noted a striking contrast in mate preference based on body size between female *F. notatus* and *F. olivaceus*. While female *F. notatus* exhibited a preference for smaller mates, their counterparts in *F. olivaceus* favored larger males. The significance of the body size was relative to the females making the choice. This divergence highlights the complexities of mate choice strategies within these species.

Although not reaching statistical significance, my data alignS with observations from *F. notatus* populations in the Pascagoula clade, where natural hybridization occurrences are rare. These populations demonstrate an ability to differentiate between conspecific mates, supporting the hypothesis that the Ouchatia population would be less discriminating. This suggests there are underlying factors influencing mate discrimination behaviors within these populations, warranting further investigation into the mechanisms driving such differences.

My findings also indicate that, on average, males tended to remain closer to the spawning media compared to females. Notably, this difference was attributed solely to sex and not to species, reinforcing the observed mating system where males typically exhibit a tendency to remain in proximity to spawning sites while females engage in roaming behavior, presumably to assess male quality. These observations contribute to the understanding of nuances of female mate choice strategies.

The discrepancy in preference between female *Fundulus notatus* and *F. olivaceus* emphasizes the complexities within the *F. notatus* complex, particularly regarding traits considered drivers of female mate choice. Studies like mine have concluded that female preferences for ecologically relevant traits are fundamental for the evolution of reproductive isolation and speciation (Head et al., 2013, Servedio 2004). These traits often serve as reliable indicators of a male's capability to acquire resources, which are essential for reproductive success. Examples of such traits include ornamental features, courtship displays, body size, parental care behaviors, genetic compatibility, and sensory signals. The preference for a specific trait can stem from a multitude of direct or indirect factors. Direct benefits may include access to certain resources, enhanced parental care,

or protection from predators. Indirect benefits may include genetic advantages associated with offspring viability (Lindsay et al., 2019). Body size has been suggested to have a significant effect on sexual selection dynamics, as mate choice may differentially affect males and females and play a role in intraspecific competition (Fairbairn, 2007). The divergence of body size preference seen in the closely related species of *F. notatus* and *F. olivaceus* suggests that the influence of body size is evolving rapidly. Understanding the role of body size in sexual selection provides insights into the prezygotic factors shaping mating systems.

Equally significant in comprehending the disparity in hybridization across contact zones is the development of an experimental framework that guarantees the reliability and reproducibility of results when investigating mate choice dynamics. I achieved this by implementing a free-choice arena setup, facilitating interactions between heterospecific and conspecific individuals of both sexes. Although choice arenas have faced criticisms in prior studies (Doughtery 2015) such as limitations in realism or being perceived as too tedious, my approach overcomes these challenges by incorporating unique features. My study included extended trial durations and high temporal resolution, made possible through the integration of AI technology. This innovative combination provides a deeper understanding of mate choice dynamics that traditional methods may not capture effectively. Moreover, my study appears to be one of the few to utilize this type of free-choice arena setup, highlighting its potential for advancing research in this field. Beyond its application in behavior and evolution studies, the broader importance of AI-type tools in various research areas cannot be overstated. The utilization of AI technology not only enhances the efficiency and accuracy of data collection but also

opens new avenues for exploration in diverse scientific disciplines. By permitting competition between males and females of both species and utilizing AI tools, I reached successful achievement of my objectives, demonstrating a method to study mate choice that integrates considerations of both morphology and behavior.

Moving forward, there are several promising avenues for further research to deepen my understanding of mate choice dynamics within the *Fundulus notatus* species complex and its implications for evolutionary and ecological processes. Understanding whether the ability for species recognition is genetically determined could provide crucial insights into the evolutionary mechanisms driving reproductive isolation and speciation processes. An approach to investigate this question is to conduct controlled crosses between different populations within the complex. By crossing individuals from distinct clades, hybrid offspring with mixed genetic backgrounds can be created. These hybrids can then be subjected to assays to assess their mate preference and species recognition abilities. If hybrids exhibit mate preferences similar to one of the parental populations, it could suggest a genetic basis for species recognition and mate choice. Conversely, if hybrids show intermediate or novel mate preferences, it may indicate a more complex genetic or environmental influence on mate choice behavior. If hybrids exhibit mate preferences similar to one of the parental populations, it could suggest a genetic basis for species recognition and mate choice. Conversely, if hybrids show intermediate or novel mate preferences, it may indicate a more complex genetic or environmental influence on mate choice behavior. The fish were raised in a controlled environment alongside their own populations in a common garden, therefore these trials marked the first instance they were exposed to individuals of the opposite species. This contrast from natural hybrid

zones offers a distinct perspective on how genetic predispositions versus how environmental factors contribute to the mechanisms underlying speciation processes. The ability of these trials to mirror observations in the field is highly relevant, as it enhances the validity of my findings. It also suggests that the mate preferences observed in these species are predisposed rather than learned, emphasizing the role of prezygotic and postzygotic factors in shaping reproductive behaviors and contributing to reproductive isolation.

In conclusion, my study provides valuable insights into the complex dynamics of mate choice behaviors within the *Fundulus notatus* species complex. Through observation and analysis, I have uncovered significant variations in female choosiness, species discrimination, and mate preferences among populations and species within the complex. These findings shed light on the intricate interplay of genetic, ecological, and behavioral factors shaping reproductive strategies and contributing to the maintenance of species boundaries. There are exciting prospects for further research to deepen my understanding of mate choice dynamics and their evolutionary implications. Investigating the genetic basis of species recognition through controlled crosses between different populations offers an opportunity to elucidate the mechanisms underlying reproductive isolation and speciation processes. By integrating genetic, behavioral, and ecological approaches, future studies can continue to unravel the complexities of mate choice within the *F. notatus* species complex and provide valuable insights into broader patterns of biodiversity and evolutionary divergence in natural populations.

# APPENDIX A: IUCAC APPROVAL

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.5997 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

**NOTICE OF COMMITTEE ACTION**

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

| | |
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| PROTOCOL NUMBER: | **15102701.3** |
| PROJECT TITLE: | **A Genomic Analysis of the Impact of Genetic Divergence, and Chromosomal Rearrangement on Introgression in Replicate Fundulus Hybrid Zones** |
| PROPOSED PROJECT DATES: | **9/2023 - 9/2026** |
| PROJECT TYPE: | **Renewal of Protocol 15102701.2** |
| PRINCIPAL INVESTIGATOR(S): | **Jake Schaefer** |
| DEPARTMENT: | **Biological Sciences** |
| FUNDING AGENCY/SPONSOR: | **NSF** |
| IACUC COMMITTEE ACTION: | **Designated Review Approval** |
| PROTOCOL EXPIRATON DATE: | **September 30, 2026** |

Samuel Bruton, PhD
Office of Research Integrity, Director

November 17, 2023
Date

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