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Sarah Stovall
The University of Southern Mississippi

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Comparing the Reproductive Fitness of *Fundulus* Hybrids and their Parental Species (*Fundulus olivaceus* and *Fundulus notatus*) within Mississippi Freshwater Contact Zones

by

Sarah Stovall

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Approved by:

Jacob F. Schaefer, Ph.D., Thesis Advisor,
School of Biological, Environmental and Earth Sciences

Jacob F. Schaefer, Ph.D., Director,
School of Biological, Environmental and Earth Sciences

Sabine Heinhorst, Ph.D., Dean
Honors College

ABSTRACT

Hybridization of closely related taxonomic groups within the Animal Kingdom has led to complex species interactions that are not well understood. Pre-zygotic or post-zygotic barriers complicate hybridization as a means to diversify; however, diverging hybrids may speciate and expedite evolution of involved taxa. This study pursues understanding of these intricate interactions using topminnow *Fundulus notatus*, *Fundulus olivaceus*, and their hybrids from the *Fundulus notatus* species complex. Persisting hybrids within riverine contact zones were hypothesized to experience barriers of hybrid breakdown from lower fitness in comparison to the pure species. Specimens were collected within four contact zones: Spring River, Pascagoula River, Tombigbee River, and Noxubee River. Each genetically differentiated specimen was phenotypically analyzed based on length, wet mass, spot density, sex, gonad mass, and eviscerated mass. Significant differences in Fulton's condition index were present in comparison of sex ($P \leq 0.001$), zones ($P \leq 0.0007$), and species across zones ($P \leq 0.04$) due to females dedicating more energy to body condition and extreme variation in contact zones, respectively. Comparisons of gonadosomatic indices (GSI) yielded significant differences between sexes ($P \leq 2.0 \times 10^{-16}$) of each species due to females investing in reproduction more than males. Spot phenotype analyses confirmed genetic analyses of each species and emphasized the intermediate traits of hybrids. Averaged indices across species did not support the original hypothesis since hybrids showed similar or increased Fulton's condition indices and GSI in comparison to the pure species. Possible explanations lie within the phenomenon of hybrid vigor, and future research should focus on replicating these results and predicting if hybrids will speciate.

Keywords: Hybridization, hybrid breakdown, fitness, reproductive barriers, gonadosomatic index, Fulton's condition index, Fundulus notatus species complex, hybrid vigor

DEDICATION

I would like to dedicate this to my parents, who always supported my aspirations and suffered through my incessant ramblings about topics in ichthyology they did not quite understand.

ACKNOWLEDGMENTS

I would like to thank Dr. Schaefer for guiding me through this process and dedicating the time to mentor me and shape my skills in research. I feel better prepared to enter the world of professional science, realize my goals, and make an impact on the world.

A final appreciation for the many *Fundulus* topminnows who sacrificed their lives for the pursuit of knowledge, may they rest in peace.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variants
GBS	Genotyping by Sequencing
GSI	Gonadosomatic Index

CHAPTER I: INTRODUCTION

Background on Hybridization

In certain instances, two distinguishable groups in close taxonomic relation (sister taxa or sister species) may successfully interbreed and yield hybrid generations (Dowling & Secor, 1997). Hybrids may be phenotypic intermediates of the parental generation, such as hybrids created by topminnow species *Fundulus notatus* and *Fundulus olivaceus,* but also may project unique character sets in allozymes, mitochondrial DNA, and/or nuclear loci (Dowling & Secor, 1997; Schaefer & Duvernell, 2013).

Due to lack of research surrounding hybridization within the Animal Kingdom, more studies seek to analyze how hybrids interact with and diversify their respective communities (Dowling & Secor, 1997; Duvernell et al., 2007). Previous studies within the Plant Kingdom introduced the concept that horizontal gene transfer from hybridization and introgression allows for stable groups to reshuffle adaptive systems and promote diversity (Dowling & Secor, 1997). When applied to animals, the role of hybridization is more restrictive but may give rise to distinct species, higher mutation rates, and increased divergence from parental forms (Dowling & Secor, 1997). Complete divergence through hybridization can occur by allopolyploidy (increase in number of chromosome sets) or by a shift to an all-female method of reproduction (parthenogenesis, gynogenesis, or hybridogenesis) (Dowling & Secor, 1997). The generation of newly diverged animal species is integral to understanding the changing world. Comprehension of emerging species and their relationships can aid in predicting environmental dynamics, evolutionary shifts, and changes in environmental relationships to humans. Thus, it is important to study mechanisms of hybridization and introgression to better grasp species interactions and their effects.

Several factors may prevent organisms from hybridizing whether they be pre-zygotic barriers or post-zygotic barriers (Duvernell et al., 2007; Vigueira et al., 2008). Within many species, pre-zygotic barriers, such as spatial or seasonal isolation, behavioral differences, morphological differences, and mate selection, may influence overall tendencies to hybridize (Duvernell et al., 2007; Vigueira et al., 2008). For example, female *Fundulus olivaceus* (blackspotted topminnow) use spots concentrated above the lateral bar as a means to recognize conspecific mates (Schaefer et al., 2012). Males have developed an exaggerated number of spots to convey high fitness and increase chances of selection (Schaefer et al., 2012). This phenomenon is considered a pre-zygotic reproductive barrier because it affects mate selection, which takes place before the production of offspring and may affect selection among other closely related species. Post-zygotic barriers refer to reproductive barriers after the zygote is developed (Vigueira et al., 2008). Examples of post-zygotic barriers include genetic incompatibility, reduced hatching success, hybrid sterility, and reduced hybrid viability (Vigueira et al., 2008). For instance, *Fundulus euryzonus* and *Fundulus olivaceus* female hybrids were found to be sterile (Schaefer et al., 2009). Both types of barriers affect fitness and ultimately should select for reduced frequency of hybridization (Schaefer et al., 2009).

Common Survival and Reproductive Barriers within Hybrid Generations

When producing hybrids, several consequences may occur that affect fitness levels and ultimately hybridization rates. Divergent species may produce inviable or sterile offspring that cannot give rise to new generations (Dowling & Secor, 1997;

Stelkens et al., 2015). Selection against hybrid generations may be considered intrinsic, low fitness regardless of environment, or extrinsic, low fitness when interacting with specific environments (Coughlan, 2022). On the other hand, hybridized offspring that can produce viable offspring may merge back with or diverge from the parental species (Dowling and Secor, 1997; Duvernell et al., 2006).

Incompatibility: No Viable Offspring

Many hybrid generations have reduced fitness due to post-zygotic mechanisms like genetic incompatibilities (Schumer et al., 2014). The recombination of parental genes may be subject to separation if the reproducing species are sufficiently divergent (Dowling & Secor, 1997; Schaefer et al., 2016; Schumer et al., 2014). Thus, the hybrid generation born from this pairing may exhibit those incompatibilities with reduced fitness or survival (Schaefer et al., 2016; Schumer et al., 2014). In this instance, selection is intrinsically against hybrids since their overall survival in any environment is affected (Coughlan, 2022). A commonly cited example of this phenomenon is two fruitfly species, *Drosophila melanogaster* and *Drosophila simulans,* that produce inviable hybrids, in part due to divergence in proteins that aid in transport of macromolecules between the nucleus and cytoplasm (Presgraves et al., 2003).

Sterility: Sex-Specific Ratios

Another outcome of hybridization is hybrid sterility. Much like inviability, sterility is a product of mismatched genes from taxonomically differing parental species (Dowling & Secor, 1997). Hybrid sterility is generally unbalanced as one sex may have greater sterility than the other (Vigueira et al., 2008). Haldane’s rule stipulates that sex-specific sterility is often seen in the heterogametic (typically male) sex rather than homogametic (typically female) sex (Schumer et al., 2014; Vigueira et al., 2008), although several instances of reversal have occurred. The heterogametic sex is more susceptible to sterility due to recessive, sex-specific deleterious effects not being buffered by another X chromosome that can carry a combatting, dominant allele (Vigueira et al., 2008). For instance, female *Fundulus euryzonus–Fundulus olivaceus* hybrids are sterile, rather than the male hybrids; however, it is not clear if males are heterogametic in this relationship (Schaefer et al., 2009). Supporting data for this specific instance identify that *Fundulus euryzonus* may have heterogametic females rather than homogametic females seen in *Fundulus olivaceus* and *Fundulus notatus*, but the sex determination mechanism is unclear (Vigueira et al., 2008). Another sex-based hypothesis, the faster-male hypothesis, predicts that male sterility factors will accumulate faster than female sterility factors, causing male-biases within the sex-specific sterility ratio (Tech, 2006). Males commonly carry a single X chromosome and express all alleles without the hinderance of recessive alleles, making males evolve faster and resulting in an isolating mechanism between sexes (Tech, 2006). A study of pupfish species *Cyprinodon variegatus* and *Cyprinodon elegans* indicates that male hybrid sterility is in part influenced by the male-faster phenomenon due to higher levels of sexual selection on males (Tech, 2006).

Hybrid Breakdown

Inverse relationships between genetic compatibility and divergence of hybrid genomes may lead to the breakdown of hybrid fitness (Stelkens et al., 2015). This breakdown is best observed after the first generation (F1) of hybrids but may be seen in the F1 generations (Stelkens et al., 2015). Hybrid crosses of seven different cichlid fish species yielded higher generations (F2 and up) that were more susceptible to deleterious homozygous traits (Stelkens et al. 2015). Furthermore, Stelkens et al. (2015) found that divergence in these later hybrid generations led to an increase in genetic distance that contributed to an increase in post-zygotic barriers.

Backcross: Merging Species

If hybrids are viable and able to reproduce, then they may backcross and merge with the parental species. Backcrossing is the process of hybrids breeding with parental species rather than hybrid generations (Stelkens et al., 2015). Gene transfer by repeated backcrossing between hybrid and parental species, also known as introgression, can fuse the hybrids back into pure species (Dowling & Secor, 1997). This is in part due to species boundaries being permeable or semi-permeable, reducing the number of reproductive barriers (Duvernell et al., 2007; Harrison & Larson, 2014). This permeability allows a bimodal system to encourage homogenization of genes across gene pools (Harrison & Larson, 2014; Schaefer et al., 2011). Generally, these bimodal systems consist of entirely pure species (Harrison & Larson, 2014; Schaefer et al., 2011). Consistent backcrossing is thought to expediate diversification within lineages that hybridize due to active gene transfer (Schaefer et al., 2011).

Species of Interest and Hybridization

The *Fundulus notatus* species complex is well-studied within several riverine contact zones to predict relationships between *Fundulus olivaceus-Fundulus notatus* hybrid fitness and the parental generation fitness. Ranges of both species extend from middle and lower parts of the Mississippi River basin to some Great Lakes drainages and along the Gulf Coast from Texas to Florida (Schaefer et al., 2009). The *Fundulus notatus* species complex is a system pertaining to the phenomenon of genetic isolation, species distribution, and hybridization within *F. notatus*, *F. olivaceus*, and *F. euryzonus* (Alldredge et al., 2011; Duvernell et al., 2006; Schaefer et al., 2011; Schaefer et al., 2016). Hence, the complex is a useful system in which to study interactions among closely related species and their hybrids (Schaefer et al., 2009). While *F. euryzonus* are not specifically observed in this study, their interactions within the *Fundulus notatus* species complex is integral to understanding different outcomes of hybridization as described earlier.

The species of interest, *F. olivaceus* and *F. notatus,* are morphologically similar in that they have a distinct lateral, black band that extends from their snout through the eye to the caudal peduncle (Braasch & Smith, 1965; Schaefer et al., 2012). *Fundulus olivaceus* is often identifiable by the presence of many black spots above this lateral bar, while *F. notatus* typically has fewer spots (Braasch & Smith, 1965; Schaefer et al., 2012). The number of spots is also a sexually dimorphic trait, with males often having more spots than the females (Schaefer et al. 2012). However, it is important to note that this characteristic is not always reliable in differentiating the species or sexes, and genetic tests are often used to confirm the species and sex (Schaefer et al., 2012).

Interactions between *F. notatus* and *F. olivaceus* can be affected by many environmental factors such as habitat preference or mate selection. For instance, habitat preference is primarily based on factors such as stream velocity, substrate, and aquatic vegetation (Braasch & Smith, 1965; Earnest et al., 2014). *Fundulus notatus* are commonly found in downstream habitats with low stream flow while *F. olivaceus* are found in headwater habitats with narrow channels, shallow waters, increased canopy cover, lower bank slope, larger substrate size, less emergent vegetation, and more woody debris (Braasch & Smith, 1965; Duvernell et al., 2007; Earnest et al., 2014). Isolation in headwaters of dendritic streams is more apparent due to an increase in distance between locations (Earnest et al., 2014). Earnest et al. (2014) studied population divergence and genetic structure in correlation with drainage distribution of the Saline River (a tributary of the Ohio River) and found that *F. olivaceus* had greater genetic isolation due to isolation by distance, while *F. notatus* did not follow this pattern. When studying mate selection, Schaefer et al. (2012) found that mate selection within *F. olivaceus* populations is heavily influenced by the number of spots above the lateral bar. Females are choosier, and typically males present the desired trait, but *F. olivaceus* present spots in both sexes indicating parallel evolution (Schaefer et al., 2012). Males increase the number of spots as they mature while females show inverse relationships with the number of spots and GSI (Schaefer et al., 2012). Differences in both species may contribute to varying levels of individual fitness and the likelihood of hybridization (Duvernell & Schaefer, 2014).

Contact zones between these two species are common throughout overlaps in their respective ranges and areas for potential hybridization (Duvernell et al., 2007). Ecological gradients within contact zones are thought to influence hybridization rates (Duvernell & Schaefer, 2014; Schaefer et al., 2016). Duvernell and Schaefer (2014) found that presence of hybrids may be affected by ecological shifts that promote phenotypic divergence, stronger pre-zygotic reproductive isolation, or reduced hybrid fitness. These shifts occur through abrupt changes from river to tributary habitats within confluences in the river continuum (Duvernell & Schaefer et al., 2014). Responses may be typical upstream and downstream species reversing through alteration of pertinent phenotypes (Duvernell & Schaefer, 2014). However, phenotypic convergence in reversed habitat associations of *F. notatus* and *F. olivaceus* was found to promote high rates of hybridization through facilitation of genetic introgression (Duvernell & Schaefer, 2014; Schaefer et al., 2016). Hybridization rates were also hypothesized to be affected by anthropogenic factors such as homogenization of habitats, endocrine-disrupting compounds, or reduction of environmental mediation of reproductive isolation mechanisms (Duvernell & Schaefer, 2014).

Within this study, reproductive fitness of *F. olivaceus*, *F. notatus*, and their hybrids was assessed across four contact zones: Spring River, Tombigbee River, Pascagoula River, and Macedonia Creek. Previous research indicates the parental species exhibit weak pre-zygotic barriers and random mating that yields hybrids (Duvernell et al., 2007). The hybrids and backcrosses appear to be viable and fertile (Viguiera et al., 2008). Our hypothesis is that hybrid generations will experience breakdown and present lower levels of fitness in response to either intrinsic or extrinsic factors. Fulton's condition indices and GSI were used to determine fitness levels and give means for comparison. We predict that hybrids across replicate populations will have a significantly lower measure of condition and fitness compared to parental species.

CHAPTER II: METHODS

Fundulus notatus and *Fundulus olivaceus* were collected from four hybrid zones that had been previously studied and in which hybridization was ongoing (Duvernell and Schaefer, 2013; Duvernell et al., 2013; Schaefer et al., 2011). The four hybrid zones were: the Spring River (below Lowell Reservoir near Joplin, MO 37.062756 -94.708679), Tombigbee River (Figure 1) (near the confluence of Kings Creek and Town Creek, in Tupelo, MS 34.234282 -88.69602), the Pascagoula River River (in the mouth of Black Creek, south of Hattiesburg, MS 37.0258 −94.7239) and Macedonia Creek (upstream from the confluence with the Noxubee River, south of Macon, MS 33.03721 -88.56239). For all four systems, the center of the contact zone was known from previous studies. It was also known that hybridization rates varied widely among contact zones. Thus, we sampled intensively in the middle of known contact zones (Figure 1) in an attempt to capture as many individuals as possible from admixed populations. Sampling was done using dipnets, and fin clips were preserved in either 95% ethanol or a high salt preservative (Seutin et al., 1991).

Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen). The EcoT22I restriction enzyme was used to construct genotyping by sequencing (GBS) libraries following Elshire et al. (2011); libraries were sequenced using Illumina HiSeq. Raw GBS data was analyzed with the TASSEL (version 5.0) pipeline (Glaubitz et al., 2014) using a *Fundulus olivaceus* genome as a reference (Brennan et al. 2014), and aligning with Bowtie 2.0 (Langmead and Salzberg, 2012). After genotyping calls were made, data were filtered for missing data (maximum of 25% missing by individual and 10% missing by locus), and only biallelic loci were retained.

We also removed any loci with excess artifactual heterozygosity (loci with >50%) (Schaefer et al. 2016). We performed an initial screen of all individuals to assess admixture status by conducting a STRUCTURE analysis (Pritchard et al., 2000), with K = 2 (average of five replicates of one million repetitions after a burnin of 100,000). In this analysis, K corresponds to the number of populations, or separate gene pools. For analyses, individuals with coefficient of membership (Q-score) > 0.97 or less than 0.03 were designated as parental species (*F. notatus* or *F. olivaceus*), and individuals with intermediate values were designated as hybrids.

Figure 1: An example of a sampled contact zone (Tombigbee River) in which Fundulus notatus, Fundulus olivaceus, and hybrids were caught.

Any *Fundulus olivaceus*, *Fundulus notatus*, and *F. notatus–F. olivaceus* hybrids caught were stored in plastic vials filled with 10% formalin (40% formaldehyde). Phenotypic traits (Figure 2) were measured for Fulton's condition index and the GSI. Only adult specimens were measured (specimens longer than 35 mm). Using Mitutoyo Absolute Super IP67 calipers, standard length (mm) was established by measuring from the tip of the snout to the caudal peduncle. An Ohaus Explorer analytical balance was used to measure wet mass (mass before dissection) in grams. Sex was documented primarily based on a sexually dimorphic characteristic, the anal fin (Schaefer et al., 2011). Male topminnows have a skinnier, sharper, and longer anal fin while females havea fatter, rounder, and shorter anal fin (Schaefer et al., 2011). Spot frequency was determined by counting distinct spots above the lateral bar on the body. Spots on the head or fins were not counted. Each specimen was then cut open laterally from the anus to the jaw and then horizontally from each side of the anus and jaw for easier access. Using dissecting scissors and tweezers, only the ovaries or testes were removed from the body cavity. The gonads were weighed to establish their mass (g) and promptly stored in a separate vial of formalin. Finally, the eviscerated mass (mass without gonads) was established. Each specimen was placed back in the vial with the corresponding gonad vial and fish number.

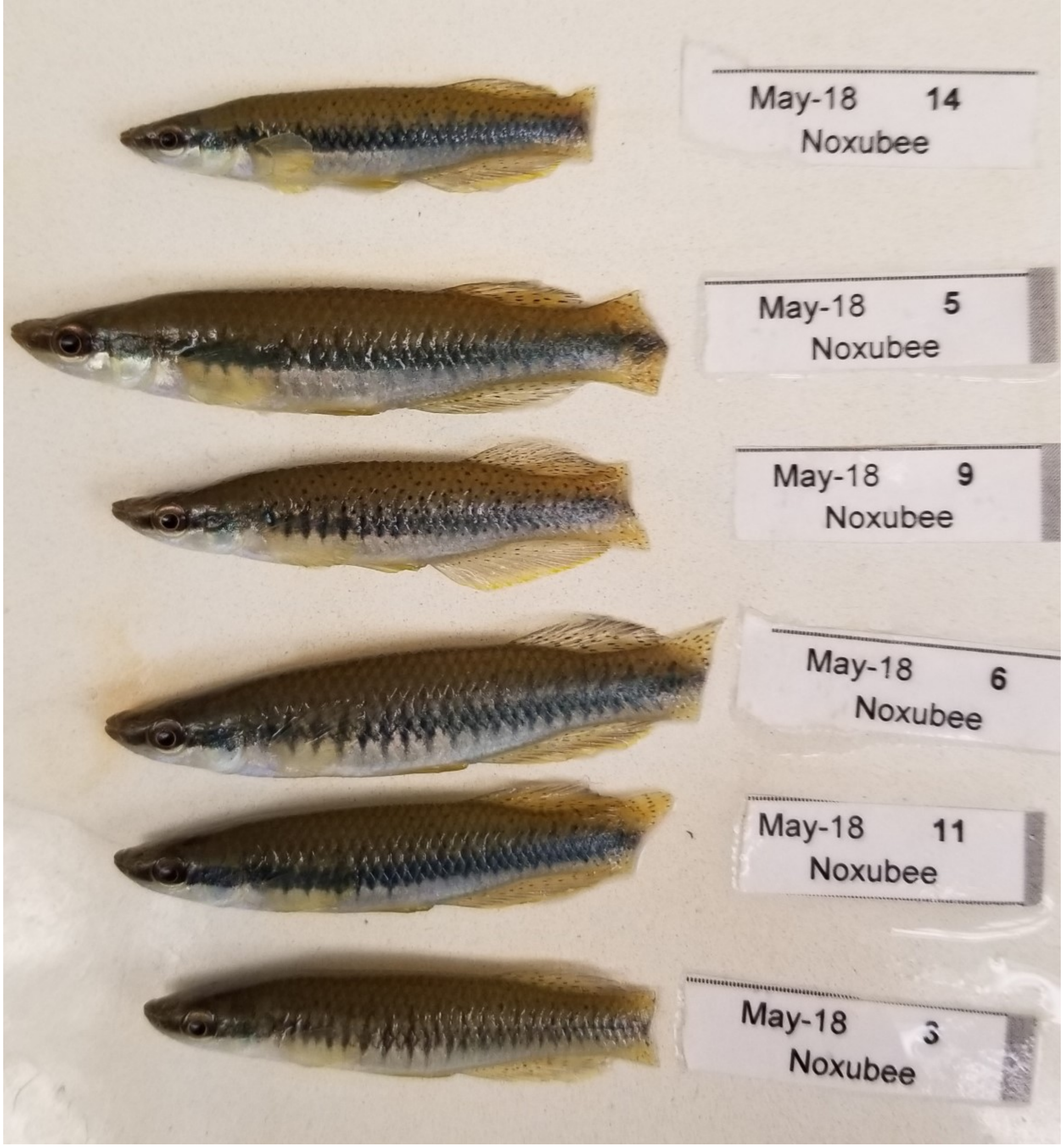


Figure 2: Variation in adult male topminnow phenotypes from Noxubee River. Differences in body size and spot count can be seen. Tags next to the fishes denote day, location, and assigned number when they were first caught.

From the data collected, condition and reproductive indices were calculated. The expression $Condition\ Index = \frac{Wet\ Mass}{Standard\ Length^3}$ was used to compare body mass indices of the parental and hybrid generations. This weight-length comparison is known as Fulton's condition index (K-factor) and is often representative of energy reserves within the body. For reproductive indices, the GSI, $Gonadosomatic\ Index = \frac{Gonad\ Mass}{Wet\ Mass}$, was calculated. The gonadosomatic index represents how much energy is invested in reproduction in comparison to overall body functions. To test the hypothesis that there is a significant difference in fitness between hybrid and pure species, averages in GSI, averages in Fulton's condition index, sex, spot phenotype, and contact zones were compared using three-way (species, sex, hybrid zone) ANOVA statistical analysis.

CHAPTER III: RESULTS

Fulton's Condition Index

There was a significant difference in Fulton's condition index between sexes, different hybrid zones, and a significant interaction between sex and hybrid zone (Table 1). There was no significant difference between hybrids and parental species (levels of species factor: *F. notatus*, *F. olivaceus*, and hybrids), or any interactions that would suggest hybrid condition was reduced in some contact zones but not others (Table 1). Across all species, the females had a higher Fulton's condition index than the males (Figure 3). *Fundulus notatus* presented a greater disparity in condition index between the two sexes in comparison to the other species (Figure 3), but this difference was not great enough to be significant in the comparison of sexes across species (Species:Sex) (Table 1). From zone to zone, species patterns were not consistent with each other and varied significantly (Figure 6).

Table 1. Three-way ANOVA results for Fulton's condition index. Significant values are symbolized by asterisks (*): ***P< 0.001, **P< 0.01, *P<0.05, .P< 0.1

Fulton's Condition Index			
	Df	F	P
Species	2	0.1	0.8
Sex	1	10.3	0.001 **
Zone	3	5.7	0.0007 ***
Species:Sex	2	.4	0.6
Species:Zone	5	2.2	0.04 *
Sex:Zone	3	0.9	0.4

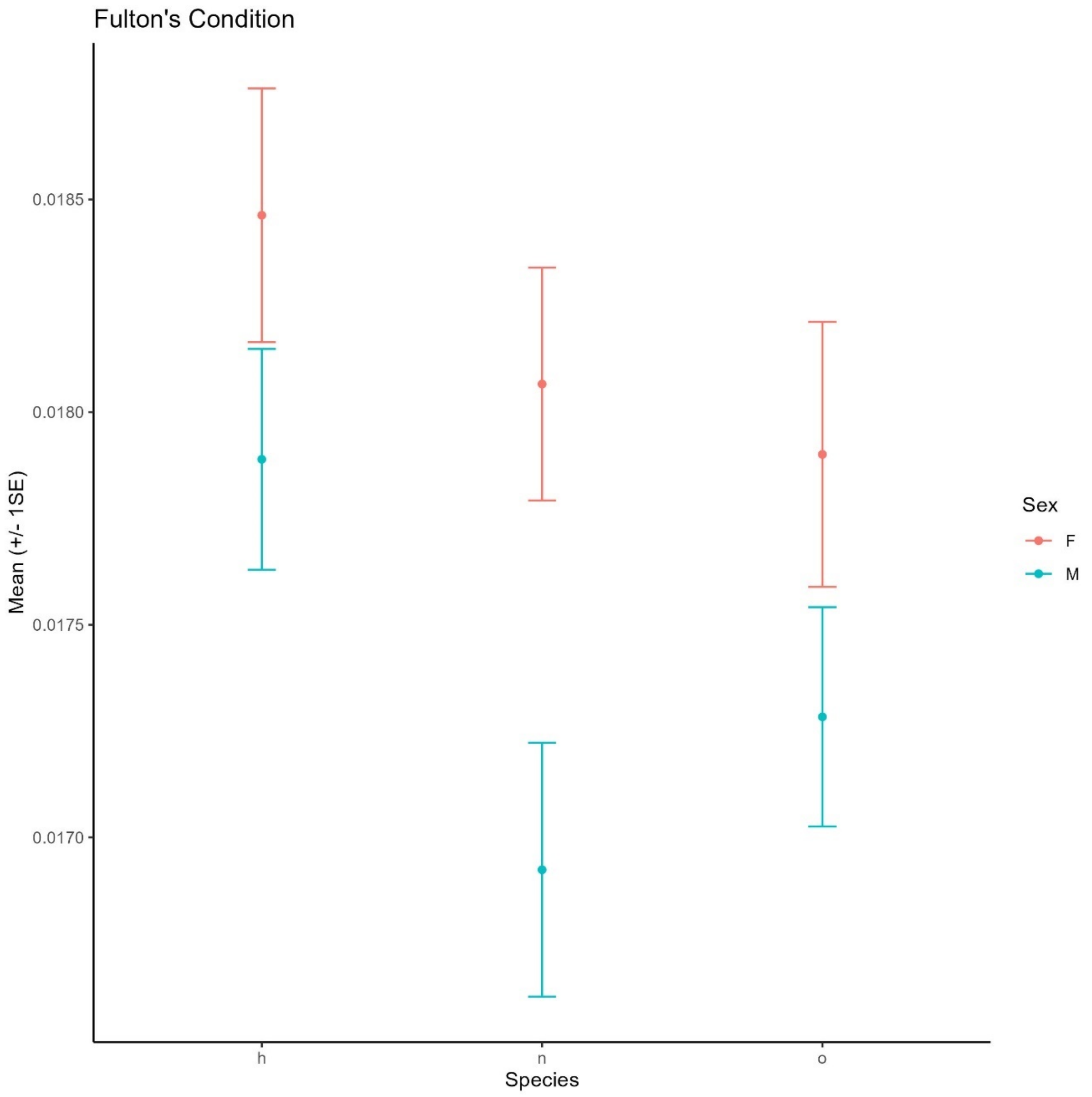


Figure 3: Fulton's condition plot. Each point is an average condition index for males (M) and females (F), respectively, of tested species: hybrids (h), *F. notatus* (n), and *F. olivaceus* (o). Standard deviation bars denote the standard deviation of the mean condition index axis.

Gonadosomatic Index

Gonadosomatic index values were significantly lower for males than for females ($P \leq 0.001$), but there were no other significant differences among species, hybrid zones, or interactions among those terms (Table 2) (Figure 4). Hybrid females had a marginally higher GSI compared to parental species (Figure 4); however, large variance and low sample size resulted in less power to detect a statistical difference ($P \leq 0.1$) (Table 2). Generally, hybrid males follow the same trend as the pure species males of having a low GSI (Figure 4).

Table 2. Three-way ANOVA results for GSI. Significant values are symbolized by asterisks (*): ***P< 0.001, **P< 0.01, *P<0.05, .P< 0.1

Gonadosomatic Index			
	Df	F	P
Species	2	1.8	0.1
Sex	1	96.7	2.0×10^{-16} ***
Zone	3	1.7	0.1
Species:Sex	2	1.8	0.1
Species:Zone	5	0.4	0.8
Sex:Zone	3	1.1	0.3

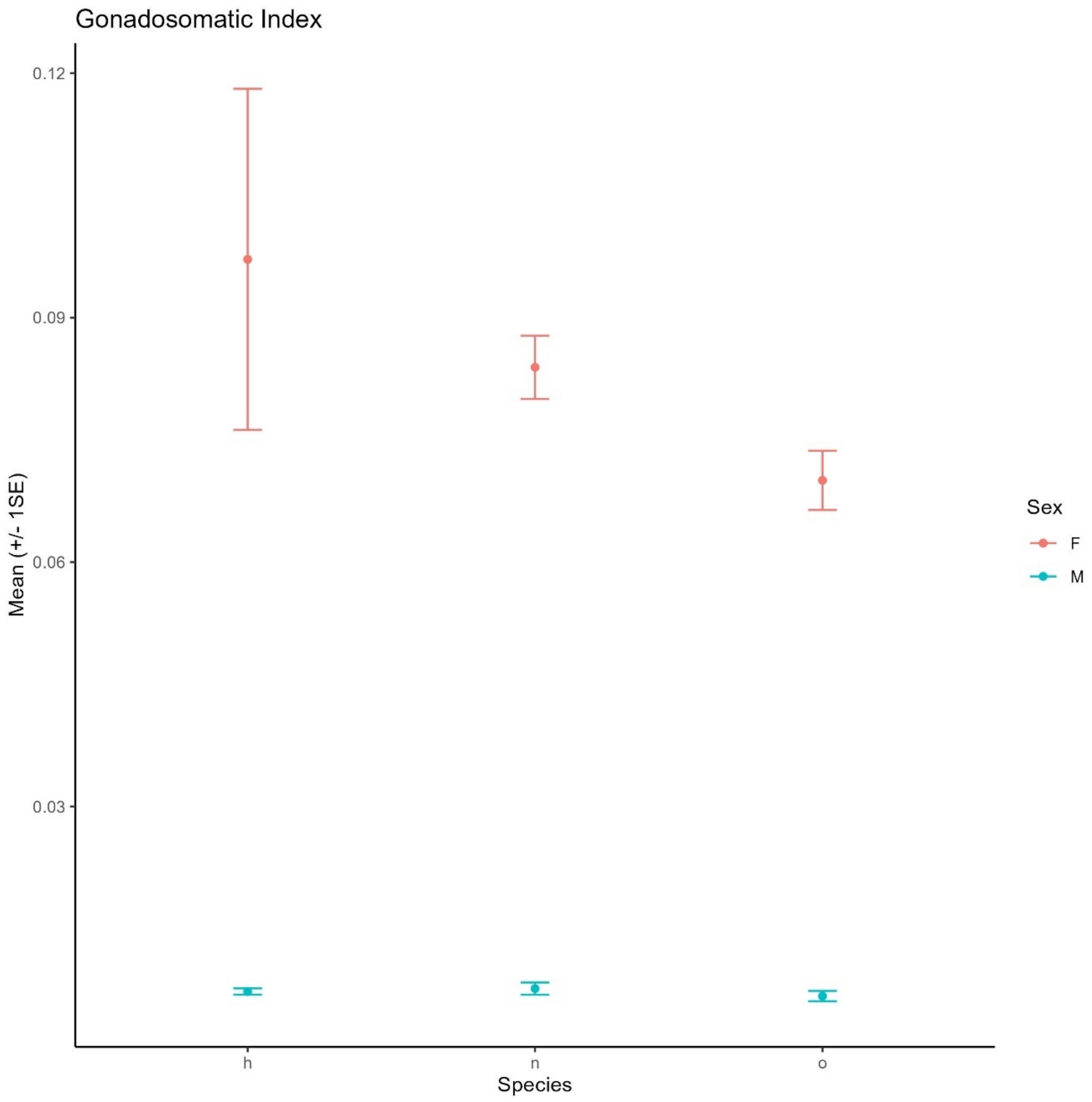


Figure 4: Gonadosomatic index plot. Each point is an average gonadosomatic index for males (M) and females (F), respectively, of tested species: hybrids (h), *F. notatus* (n), and *F. olivaceus* (o). Standard deviation bars denote the standard deviation of the mean gonadosomatic index axis.

Spot Phenotype

Across species, spot values as well as spot density across zones were highly significant (Table 3). Distinction of species is supported by high average spot count for *F. olivaceus*, low spot count for *F. notatus*, and intermediate spot count for hybrids (Figure 5). Marginally significant values for Species:Sex and Species:Zone are noted as small differences (Table 3). Different zone structures affect species populations and thus overall spot density (Figure 6). Male spot count in *F. olivaceus* is greater than female spot count, but *F. notatus* and hybrids do not present this trend (Figure 5).

Table 3. Three-way ANOVA results for spot phenotype. Significant values are symbolized by asterisks (*): ***P< 0.001, **P< 0.01, *P<0.05, .P< 0.1

Spot Phenotype			
	Df	F	P
Species	2	160.2	2.2×10^{-16} ***
Sex	1	0.2	0.5
Zone	3	7.3	9.1×10^{-5} ***
Species:Sex	2	2.8	0.05 .
Species:Zone	5	2.1	0.05 .
Sex:Zone	3	0.5	0.6

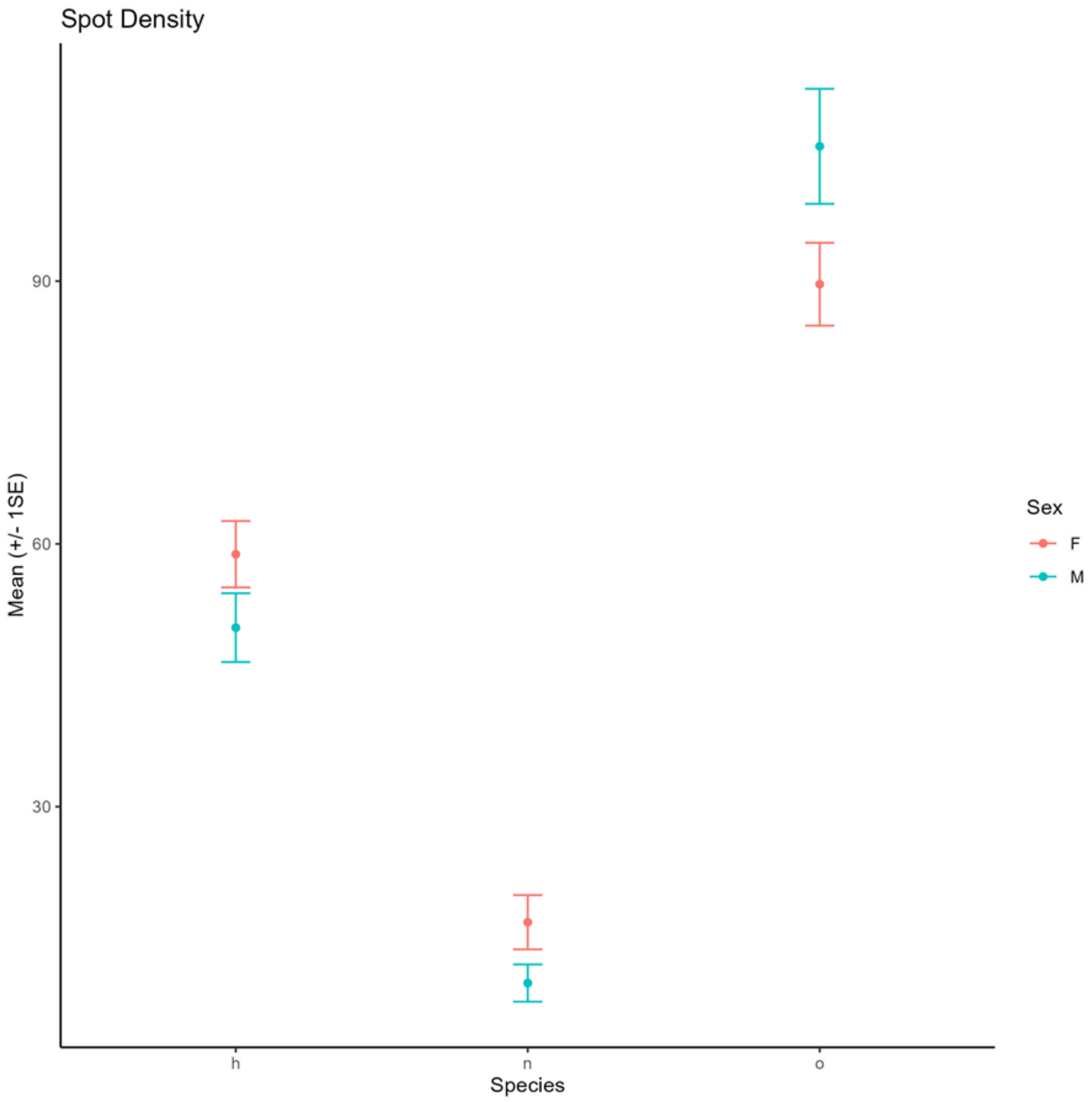


Figure 5: Spot density plot. Each point is an average spot phenotype count for males (M) and females (F), respectively, of tested species: hybrids (h), *F. notatus* (n), and *F. olivaceus* (o). Standard deviation bars denote the standard deviation of the mean spot phenotype axis.

Genetic Data Across Contact Zones

STRUCTURE analysis of all individuals sampled (adults and juveniles) revealed large differences in the rates of hybridization among the four areas. The Tombigbee (20 *F. olivaceus*, 158 hybrids, and 58 *F. notatus*) and Noxubee (12 *F. olivaceus*, 96 hybrids and 45 *F. notatus*) Rivers were both dominated (>60% of individuals) by hybrids (Q scores <0.97 or > 0.03). The Spring River (42 *F. olivaceus*, 30 hybrids and 39 *F. notatus*) and Pascagoula (57 *F. olivaceus*, 3 hybrids, and 81 *F. notatus*) Rivers each had substantially fewer (27% and 2%, respectively) hybrids (Figure 6). Only the Spring River seemed to have a large number of putative F1 hybrids. The Tombigbee and Noxubee River systems both clearly had more extensive back crossing with fewer F1 hybrids. The only hybrids sampled in the Pascagoula River appear to be likely first generation back crosses with admixtures of ~0.75. Given the presence of F2 and backcrossed hybrids, the data supports the viability of F1 hybrids, consistent with previous studies.

Only adults were analyzed for each measured variable; thus, 281 specimens were measured in total. Of the dissected individuals, 107 adults were collected from the Spring River, 57 adults were collected from the Pascagoula River, 59 adults were collected from the Tombigbee River, and 58 adults were collected from the Noxubee River. Some adults were not measured due to desiccation or misplacement.

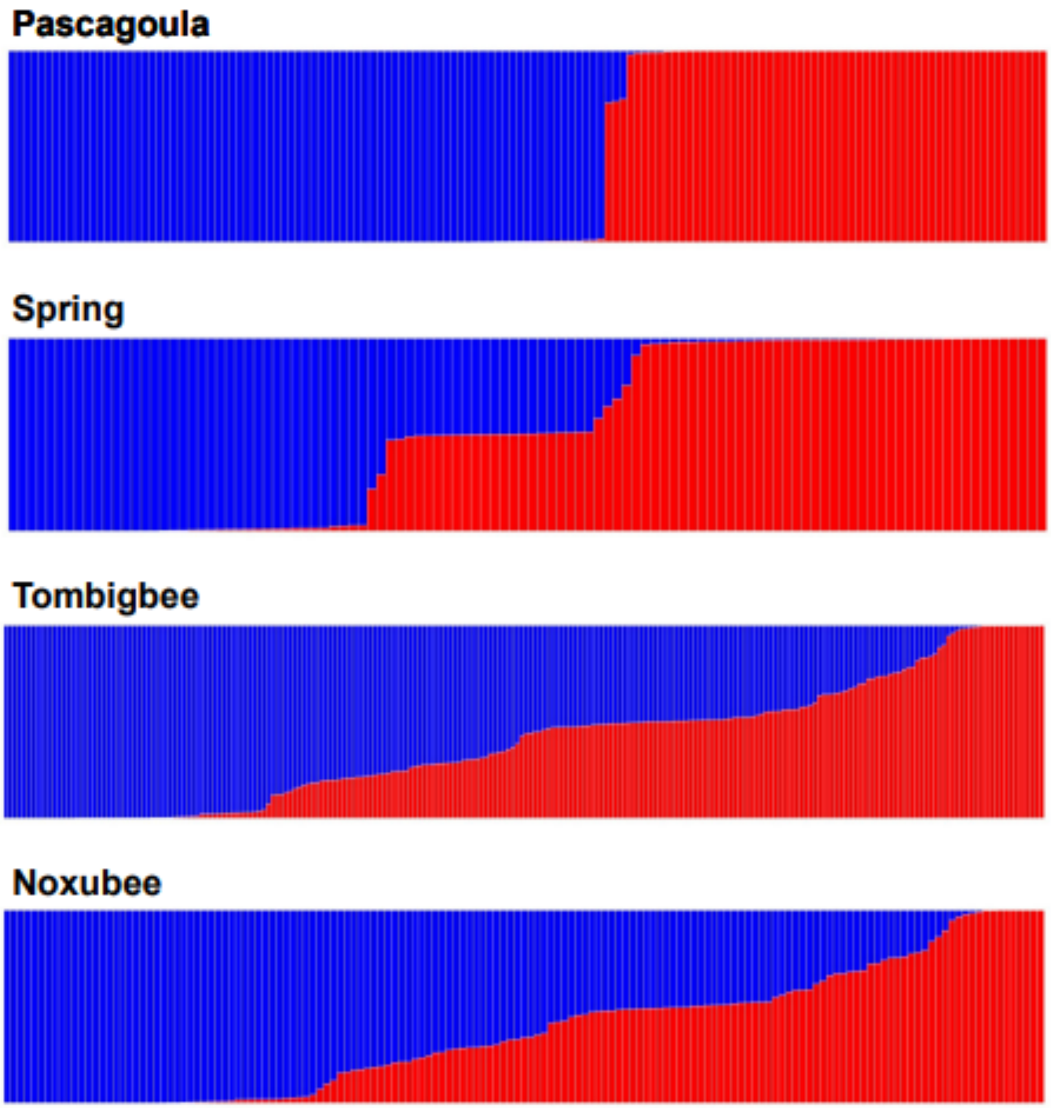


Figure 6: Contact zone species structure (K=2) plots. All red or all blue bars are representative of pure species while red and blue bars represent estimated admixture proportions between either *F. notatus* or *F. olivaceus* for all individuals sampled in hybrid zone.

CHAPTER IV: DISCUSSION

Although our hypothesis predicted that *Fundulus notatus* and *Fundulus olivaceus* hybrids would experience lower condition and reproductive fitness as a result of hybrid breakdown, the results contradict this expectation and lead to some interesting new hypotheses. Previous research suggests that there is weak pre-zygotic isolation and that hybrids are fertile (Duvernell & Schaefer, 2014). Data from this study suggests that hybrids present similar or higher fitness to the pure species. Nevertheless, more hybridization between the species has not been observed and a few questions are left to be answered.

Analyses of Species and Sex Differences

General differences among species are adequately defined through significant differences in spot phenotype (Table 3) and depictions of spot density (Figure 5). Trends of *F. olivaceus* having the greatest spot density as well as males prioritizing this trait is synonymous with phenotypic analyses that attribute spots to sexual dimorphism (males typically have more spots) and its role in mate selection (Schaefer et al., 2012). Given that *F. notatus* have the lowest number of spots, hybrid generations have intermediate spot density (Schaefer & Duvernell, 2013). Significant differences in spot density across zones identifythe variability in habitat and how one species may predominate in one area over another. This can be seen with *F. notatus* and *F. olivaceus* preferences for downstream habitats and headwaters, respectively (Braasch & Smith, 1965; Duvernell et al., 2007; Earnest et al., 2014). The spot density phenotype lends support to the results from the original genetic analyses and effectively differentiates the species.

The most significant value within the GSI analyses was obtained within comparisons of sex (Table 2). Females had a greater GSI than males, largely due to higher reproductive investment in ovaries (Figure 4). Males often dedicate energy to being selected as a mate, as shown by *F. olivaceus* males, by investing in high spot density to convey high fitness to choosy females (Schaefer et al., 2012). Therefore, not much energy is allocated to GSI (Schaefer et al., 2012). Female *F. notatus* had higher GSI than *F. olivaceus,* not supporting the hypothesis surrounding habitat preference. Duvernell and Schaefer (2014) found that *Fundulus* in downstream habitats vulnerable to environmental stressors increase energy spent on condition and subsequently gonad mass. Thus, *F. olivaceus,* which prefer downstream habitats, would present a higher GSI than *F. notatus*. However, this is not seen within the data and may be best explained by Duvernell & Schaefer's (2014) study in which *F. notatus* showed the same trend. *F. olivaceus* had a more tributary-like phenotype along the riverine system, so they only showed increase in Fulton's condition index rather than GSI. Thus, *F. notatus* were noted to have a higher GSI because they focus more on reproduction rather than growth (Duvernell & Schaefer, 2014).

Fulton's condition index analyses were highly significant across zones, species:zone, and sex (Table 1). Zones were highly significant most likely due to ecologically different habitats affecting condition indices of the residing species. Duvernell and Schaefer's (2014) study on contact zone dynamics between *F. notatus* and *F. olivaceus* introduced the idea that populations that are subjected to high predator pressure and low food availability invested less in body condition unlike the upstream populations. Since *F. notatus* prefer downstream habitats, condition indices are expected to be lower than for upstream *F. olivaceus* populations. Low male condition in *F. notatus,* as well as similar female condition to *F. olivaceus* partially support this claim and explain how zone greatly affects condition (Figure 3). Furthermore, the significant difference between sexes follows a trend that females have higher condition indices than males (Figure 3). This may also be due to females accumulating greater mass by dedicating energy to large ovaries as seen in studies with other fish species such as hakes (*Merluccius merluccius*) (Costa, 2013).

Hybrid Fitness and Vigor

While some of the data confirms known information about species differences based on preferred habitat, energy investments, phenotype, and sex, factors not supporting the original hypothesis lie within the condition and GSI of hybrids. Hybrids maintained similar if not greater Fulton's condition indices in comparison to the pure species (Figure 3). In terms of GSI, they have extremely similar indices to the pure species and a slightly higher GSI for females (Figure 4). With such unexpected results, a new hypothesis about hybrid vigor should be considered.

Hybrid vigor is best explained through the alternative dominance hypothesis (Crow, 1948). Under this hypothesis, additive recessive alleles contribute to deleterious effects as seen in hybrid breakdown (Crow, 1948). However, hybrid vigor is predicted to increase if the deleterious recessive allele from one parent is negated by the dominant allele of the other parent (Crow, 1948). A study of the replacement of the Pecos pupfish (*Cyprinodon pecosensis*) population by its hybrids with the sheepshead minnow (*Cyprinodon variegatus*) introduces the potential effects of hybrid vigor (Rosenfield et al., 2004). For instance, hybrids between the two species were studied for growth rates and swimming performance given negative correlation with predation risk (Rosenfield et al., 2004). Rosenfield et al. (2004) found that F1, backcross hybrids, and *C. variegatus* all had higher growth rates and swimming performance than *C. pecosensis*. Therefore, genetic synergy pushed ecological advantages of the hybrids to the point of outcompeting *C. pecosensis* (Rosenfield et al., 2004). Now, the *C. pecosensis* populations are threatened by this expression of hybrid vigor and are subject to risk of population decline (Rosenfield et al., 2004).

While this system dramatically places hybrids as superior, there are some similarities with the *Fundulus notatus* species complex. Stabilizing contact zones within the *F. notatus* species complex may have reduced pre-zygotic and post-zygotic barriers as seen in the aforementioned *Cyprinodon* complex (Rosenfield et al., 2004). Decrease in such barriers allow hybrids to have similar or greater fitness across the complex due to vigor (Rosenfield et al., 2004). Hybridization between the species could increase since it is beneficial to allocate energy to fit offspring. If hybridization continues, the hybrids may fully speciate over time and either coexist with the pure species or outcompete them.

Future Steps

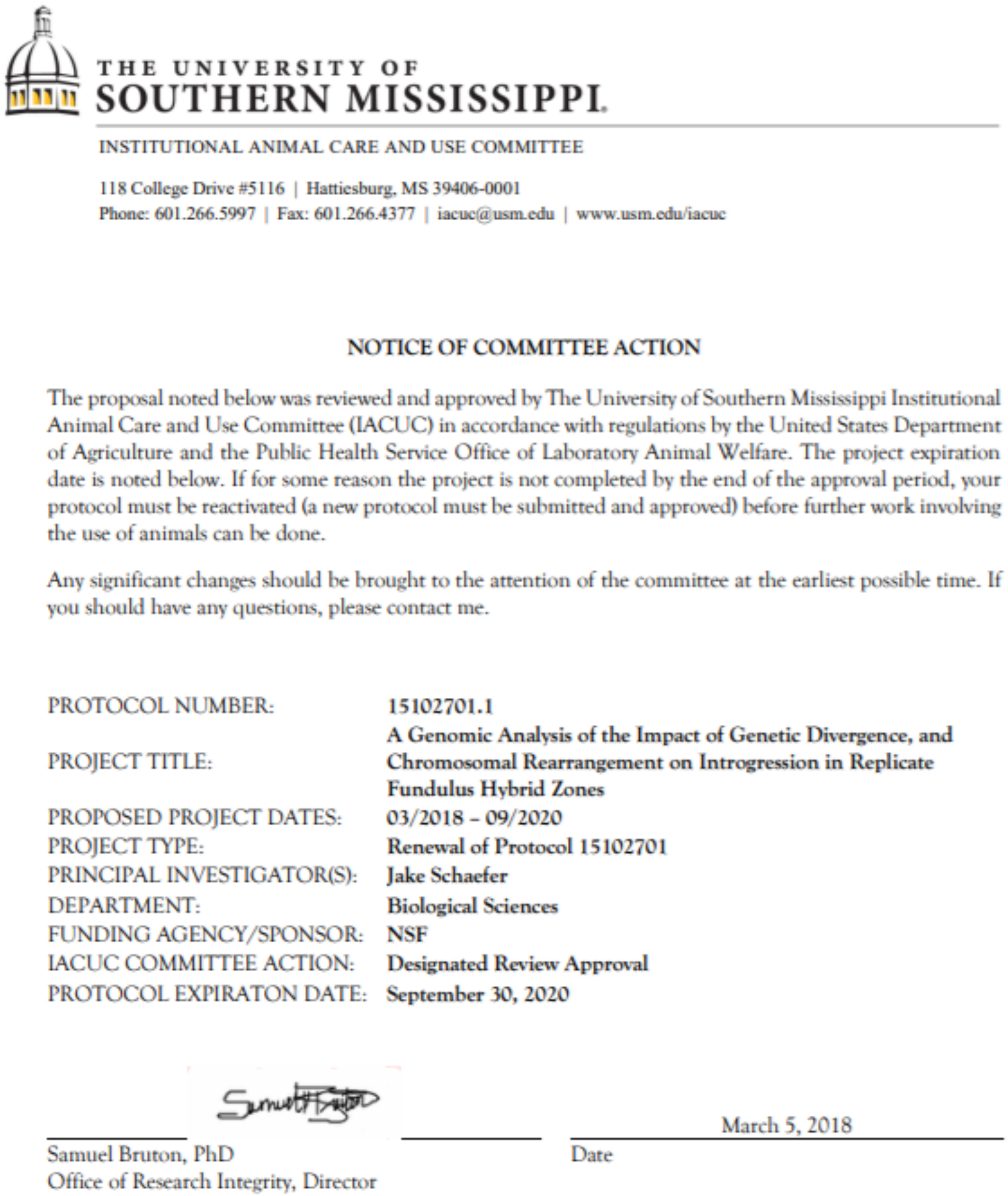
If hybrids within the *Fundulus notatus* species complex are projected to endure based on the data obtained in this study, then competition rates among the species will increase and may possibly lead to the removal of one or possibly both of the pure species within these contact zones. Areas where one pure species is dominant over the other may cause the lower populated species to suffer from hybrid vigor. This can be seen in the Cyprinodon example, in which *C. pecosensis* is outcompeted by the hybrids and *C. variegatus* (Rosenfield et al., 2004). Conversely, hybrids may coexist with the pure species and resource partition effectively; however, having similar ecological niches suggests promotion of competition. These areas should continue to be observed and monitored for change in future research.

While data points to hybrid vigor in *Fundulus notatus*-*Fundulus olivaceus* hybrids, it is important to note the overall sample size collected as well as inconsistencies across sample sizes and zones. Sample sizes were smaller than intended, given time restrictions within the lab and field, and varied drastically between Spring River and the other three zones. Therefore, results across zones and species are not properly weighted in terms of fish and species number bias. Moreover, clutch size and egg size were not taken into account when calculating the GSI, so female energy allocation to size and number of eggs cannot be factored in further reproductive fitness comparisons across species. Future studies should increase sample size and invest time in analyzing eggs to expand collected data and provide more reliability. With biases in mind, these modified studies should strive to address hybrid vigor and potential detrimental effects on one of the pure species.

Conclusion

The *Fundulus notatus* species complex is a helpful representation of hybrid interactions with pure species. Results favoring hybrid Fulton's condition index as well as GSI did not support the original hypothesis and gave insight into the possibility of hybrid vigor. These mechanisms allow for further investigation of hybridization within the Animal Kingdom and how diversification can stem from these interactions.

APPENDIX A: IACUC APPROVAL LETTER



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.

PROTOCOL NUMBER:	**15102701.1**
PROJECT TITLE:	**A Genomic Analysis of the Impact of Genetic Divergence, and Chromosomal Rearrangement on Introgression in Replicate Fundulus Hybrid Zones**
PROPOSED PROJECT DATES:	**03/2018 – 09/2020**
PROJECT TYPE:	**Renewal of Protocol 15102701**
PRINCIPAL INVESTIGATOR(S):	**Jake Schaefer**
DEPARTMENT:	**Biological Sciences**
FUNDING AGENCY/SPONSOR:	**NSF**
IACUC COMMITTEE ACTION:	**Designated Review Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2020**

Samuel Bruton, PhD
Office of Research Integrity, Director

March 5, 2018
Date

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