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Habitat preference of *F. notatus* and *F. olivaceus* from a reversed hybrid zone

by

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ABSTRACT

Understanding the mechanisms driving habitat preference throughout an organism's life opens doors to the further understanding of the origins of diversity. Two species of minnow, *Fundulus notatus* and *Fundulus olivaceus*, are ideal for ecological research on habitat preference. Ordinarily, *F. notatus* and *F. olivaceus* display habitat preferences of downstream and upstream, respectively, with minimal coexistence at confluences. However, in some drainages, these preferences are flipped, like those in the Tombigbee River basin. Members of both species were collected from the Tombigbee River, tagged with species and sex specific colored elastomer marks, and placed in either a homogeneous control or heterogeneous mesocosm designed to mimic an upstream and downstream habitat. Both mesocosms then had a camera placed over each pool (three upstream and downstream pools in each treatment). Pictures were taken every 30 seconds through two hour trials. Images were processed through an artificial intelligence (AI) system (Tensorflow) trained to recognize fish and colored elastomer tags. After processing, images were manually reviewed to assess AI accuracy and make any necessary corrections. Results showed coexistence was higher within homogeneous than in heterogeneous treatments. Both sexes of *F. notatus* and female *F. olivaceus* displayed a strong preference for the downstream orientation in the heterogeneous treatment, whereas *F. olivaceus* males showed a weak preference for the upstream orientation. Both species showed weaker preference in the homogeneous treatment. All categories involving treatment as a factor were determined to be statistically significant. The original hypothesis that the species within the heterogeneous treatment would show a higher preference than those in the homogeneous treatment was supported.

Keywords: niche, habitat preference, *Fundulus olivaceus*, *Fundulus notatus*, coexistence, mesocosms

DEDICATION

To my parents, Sam and Lisa, along with my friends and family that have shown me support not just during the thesis process, but over these past four years.

Dad, thank you for your constant encouragement. I would not have the goals or accomplishments that I have today if it weren't for you always pushing me to do the "right thing."

Mom, thank you for your support, but also for teaching me to strive to be a well-rounded person outside of academics (and for providing frequent comedic relief).

To my friends, thank you for always being a shoulder to lean on, motivators, and accomplices in procrastination.

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CHAPTER I: INTRODUCTION

Niche

Every population has a preferred set of abiotic and biotic conditions in which it is most likely to thrive, create progeny, and prevail for many generations. This specific habitat preference is referred to as the population's niche (Wiens et al., 2010; Hutchinson, 1957), and niche conservatism is the tendency of a species to preserve their fundamental niche over time (Weins & Graham, 2005). Due to this understanding of niche conservatism, when a species diverges, it should retain the preference for its original niche, which is why closely related species are typically observed as having similar habitat preferences. The idea that the distribution of a species is related to its ecological preferences is over a century old (Grinnell, 1917), and that century has allotted time for various definitions of the term "niche" to be proposed. Smith (1966) simplifies the concept of the niche by proposing that individuals will be most adapted to the specific habitat, or niche, in which they were raised due to surviving in that niche throughout their youth.

There are several subsets of concepts within the umbrella term niche that include the Grinellian, Eltonian, fundamental, and realized niche. The Grinellian niche refers to the non-competitive, abiotic environmental components of a habitat (Grinnell, 1917). The Eltonian niche refers to the resource-consumer dynamic involving biotic factors of an environment or, broadly, the role of a species in a given ecosystem and its relationship to food and enemies (Soberón, 2007; MacArthur, 1967; Elton, 1966). The fundamental niche is the set of abiotic conditions in which a species is able to survive, or all of the space a species could potentially occupy. The realized niche takes not only the abiotic components of a habitat into consideration, but also the presence of other species and the interactions this coexistence creates (Hutchinson, 1957). In other words, the realized niche is the space a species actually occupies within the whole spectrum of the fundamental niche.

Sometimes, we see anomalous cases where closely related species or populations have clearly changed their niche, such as niche reversals. These cases give us valuable insight into the evolutionary processes involved in niche modifications. This is the case in a study by Duvernell & Schaefer (2013), where the normal distribution of two species of topminnows, *Fundulus notatus* and *Fundulus olivaceus*, is switched. In a normal coexisting distribution, *F. notatus* resides in backwaters and margins of large rivers, whereas *F. olivaceus* resides in high-gradient headwaters of streams (Braasch & Smith, 1965). However, in a few drainages, this pattern is reversed, with *F. notatus* residing upstream and *F. olivaceus* residing downstream.

Another example that exhibits deviations in expected niches can be seen in a study by Remsen & Cardiff (1990), who examined the population of *Chamaepetes goudotii* (a bird known as the sickle-winged Guan). Populations of this species are typically found at low elevations up to 2,100 meters. However, a recently discovered population has a distribution range of 3,000 to 3,300 meters. To explain this discrepancy, Remsen & Cardiff (1990) hypothesized that the expected distribution and the anomalous distribution were once continuous, and the two observed distributions are remnants of a once uninterrupted distribution. Although Remsen & Cardiff's (1990) results were deemed "unsatisfying" at explaining these niche reversals, their discovery is a significant one in that the authors only cite two other cases of niche reversal in birds in the world.

This lack of reversal cases is universal and, in rare cases where niche reversals are recorded, they are anomalies and not fully understood by the science community (Martins et al., 2018). In both of these cases, there are well documented species that specialize in one region of a habitat versus the other (e.g., high-low elevation and up-downstream) and, in general, very few species are generalists enough to occupy all regions of a habitat. The lack of documentation speaks to the rarity of niche reversals within species.

Overview of Habitat Preference

Broadly defined, habitat preference is an organism's evolved response towards choosing a specific niche that maximizes its fitness over the other available niches. More specifically, it is the evolution of any change in bias for the environment in which a species, who is able to disperse, chooses to reproduce and is always assumed to influence the fitness of that species (Berner & Thibert-Plante, 2005). Habitat preference can work as a form of reproductive barrier between species, and therefore speciation, and displays the inherent needs of a species (Berner & Thibert-Plante, 2005; Rice, 1987; Aarts et al., 2007). A high-level of variation in habitat preference between populations maintains a high level of variability in the genes affecting viability (Smith, 1966; Rausher, 1984). Habitat preference is most often thought of as innate or acquired, meaning it can either be passed on through generations genetically or through learning, usually at an early age (Beltman & Metz, 2005).

Innate and Acquired Mechanisms of Habitat Preference

Mature individuals often select habitats similar to those in which they were raised, aligning with what their parents preferred (Beltman & Metz, 2005; Davis, 2008; Smith, 1966). As previously mentioned, innate and acquired mechanisms of habitat biases are the genetic and learned components of habitat preference, respectively. Thus, if it is an innate preference for the niche, parents will pass on those favorable genes to their offspring, which will increase the fitness for that niche, and the cycle will continue with each generation. Likewise, if the niche is an acquired preference, the offspring will be exposed to the appropriate cues during the developmental phases in their life. In the occurrence of both mechanisms, the preference may lead to non-random mating with individuals who prefer the same habitat, which leads to the offspring preferring the same habitat as their parents. Habitat selection is also able to facilitate speciation by reproductive isolation if an individual chose to reside in a new environment that is unfamiliar to the population (Beltman & Metz, 2005). Habitat preference is not defined solely by one mechanism or another, as it can be a combination of both (Takahashi & Masuda, 2019).

According to Davis (2008), there are three mechanisms that could take place in the natal habitat that would affect habitat preference at the time of dispersal. The first mechanism is the quality of the natal habitat. If the quality is poor, this would be deleterious to the physiological condition of individuals, which would, in turn, cause them to prefer poorer quality habitats at the time of dispersal (Davis, 2008). The second mechanism relates to the natal habitat selecting for advantageous traits that are not selected for in non-natal habitats. If the natal habitat selects for a specific trait that other habitats do not, it would be disadvantageous to the species to disperse to the non-natal habitat because the species would be decreasing their fitness (Davis, 2008). Unlike the first two innate mechanisms, the third involves acquired preferences such as imprinting (Davis, 2008).

Imprinting is described as the exposure to stimuli (often in the form of an object, habitat, or organism) early in life during a developmental stage where the individual is most susceptible to learning (Lorenz, 1935; Arvedlund et al., 1996). Imprinting causes an effect on the ecological, sexual, or filial preferences that the individual has as an adult (Arvedlund et al., 1999; Berner & Thibert-Plante, 2005; Bolhuis, 1991; Stamps, 2001). It is a mechanism that enhances a species' fitness and reproductive ability by residing in the habitat in which the individual was raised, or a habitat with similar characteristics (Dixson, 2013; Stamps, 2001). In their study, Arvedlund et al. (1999) found that olfaction, water gradient, and chemical cues can all be bases for imprinting, with select species having a higher affinity for imprinting than others. As seen in the host preference of anemonefishes, imprinting can act as an acquired mechanism of habitat preference, in conjunction with an innate, or genetically predetermined, mechanism (Arvedlund et al., 1999). Likewise, imprinting cues are demonstrated in coho salmon homing. Each river has a distinct scent; the salmon imprint on these chemical cues at a critical period at around 16-18 months of age, and then recall this memory during spawning migration to revisit their natal habitat (Tilson et al., 1993). A study with male pied flycatchers suggests that imprinting can lead to an increase in reproductive isolation and coexistence of the two species experiencing secondary contact if the species imprints on a habitat differing from their own (Vallin & Qvarnström, 2011). Secondary contact can be defined as an instance where two species in allopatry are geographically reunited. If these species did not completely develop reproductive isolating mechanisms while in allopatry, then secondary contact will most likely result in hybridization (Grant & Grant, 2009)

The given examples all exemplify ecological imprinting, which is the formation of habitat, food, or other preferences by means of being confined to a distinct diet as a juvenile or being exposed to specific habitat conditions (Arvedlund et al., 1999). According to Arvedlund et al., ecological imprinting follows the same criteria as the classical imprinting criteria that were set forth by Immelmann (1975): (1) imprinting takes place only during a sensitive, or developmental, period during the individual's life, (2) imprinting is irreversible, (3) imprinting requires the learning of species-specific characteristics, (4) imprinting may be finalized before the individual needs to recall upon it.

Conclusion

Habitat preference is a delicate natural phenomenon that is potentially molded by a multitude of factors. A species' niche has the potential to be shaped by various innate and acquired mechanisms. In rare instances, niche deviations are discovered within a species, which suggests that habitat preference is not fully controlled by innate mechanisms alone. As previously mentioned, this is exemplified in localities of *Fundulus notatus* and *Fundulus olivaceus* (Duvernell & Schaefer, 2013), where habitat preference for both species is reversed. Due to the rare nature of niche reversals, these occurrences could potentially provide insight on the mechanisms of habitat preference.

In this experiment, fish were sampled from the Tombigbee River in Alabama, which is a known reversed hybrid zone for *F. olivaceus* and *F. notatus*. Fish were placed in mesocosms intended to simulate a neutral habitat with a standard upstream and downstream orientation. In doing this, I was able to track whether the fish retained their original habitat preference, adopted a new preference, or showed no preference. The null hypothesis of this experiment predicted that neither *F. olivaceous* nor *F. notatus* would show a preference for an up or downstream orientation. The alternative hypotheses predicted both species would exhibit a preference, whether that be a reversed preference or retaining their original habitat preference.

CHAPTER II: METHODS

Fish Collections

Individuals of both species were collected by dip net from locations within the Tombigbee River basin outside of known hybrid zones, and where populations have previously been studied and genotyped (Schaefer et al., 2011). In the Tombigbee River system, *Fundulus notatus* is found in headwater streams down through medium rivers, whereas *F. olivaceous* is found in medium to larger rivers downstream. Documented coexistence and hybridization in the Pascagoula occurs at the confluences. Fish were transported to Lake Thoreau Environmental Center where the two species were housed separately in 2000 L outdoor holding tanks for two days. During this period, naturally occurring food was supplemented with freeze dried bloodworms and fish were observed for any signs of stress due to capture and transport. Once fish were observed actively feeding with no signs of stress, they were individually anesthetized using tricaine methanesulfonate (MS-15102701.1) and given a sex and species-specific elastomer tag mark. *Fundulus notatus* were marked yellow (male) and pink (female), and *F. olivaceus* were marked red (male) and orange (female) dorsally, just anterior to the dorsal fin. Preliminary work with fishes marked in this way confirmed that we could easily identify species and sex from pictures taken over mesocosms.

Mesocosms

Experiments were conducted in two stream mesocosms (Matthews et al., 2006) each comprised of six pools (circular tanks, 183 cm in diameter) connected in series by five shallow and rectangular riffles (43 cm wide and 183 cm long, Fig. 1). Mesocosms were supplied by ground water, and experienced ambient photoperiod (under 50% shade cloth). The two mesocosms were modified into two treatment levels: 1) a stream gradient with distinct up and downstream habitat (heterogeneous), 2) a control that was homogeneous throughout (hereafter homogeneous). Within each mesocosm, three tanks were designated upstream and three downstream corresponding to the direction of flow from small recirculating pumps. For the control treatment level, pools and riffles were the same throughout with sand and gravel as the sediment, no additional canopy cover, uniform water depth of 60 cm in pools and 15 cm in riffles, negligible flow from a small recirculating pump, and ambient temperature (upstream – 26.8°C ± 0.09 SE, downstream – 26.9°C ± 0.09 SE). The heterogeneous treatment level was modified to have different habitats up- and downstream to mimic the ends of a natural stream gradient (e.g., upstream habitat was shallower, with larger substrate, greater canopy cover, and higher flow in riffles). Within this treatment level, the upstream section had cobble and gravel in the shallower riffles, decreased depth overall (< 40 cm in pools, as low as 5 cm in riffles), increased canopy cover from camouflage netting installed four feet above the water-surface, and increased flow rates (high flow recirculating pumps generating flows of up to 15 cm/s) in riffles. The downstream section was comprised of greater depths (60 cm), sand as the primary substrate, no canopy cover, and negligible flow rates.

For each trial, 10 males and females of both species (40 total individuals), were randomly selected from holding tanks and distributed among the six pools within a mesocosm. This density (6.6 fish per ~1500 L pool) was chosen as it was well below densities where previous experiments had detected no density dependent effects with ambient food available (Schaefer et al., 2016). Fish were allowed to acclimate and move around mesocosms for a minimum of 24 h before observations began. After the acclimation period, cameras mounted above each pool began an observation period. Observation periods lasted 7 hours and always occurred from 9 am to 4 pm. During observation periods, cameras took one picture every 30 seconds. For a complete trial, a treatment level was observed six days with at least one full day between observation periods. Clear skies were needed for optimal data collection (clouds resulted in glare; precipitation of any kind disturbed the water surface reducing image usability), so trials were timed based on local weather. Once each treatment level had been observed a minimum of six times, fish were removed and placed in the second set of mesocosms and a new trial was initiated.

Data Collection and Analysis

After each observation period, all images were archived and named to identify the date, time, and pool (1-6) where it was taken. Each trial was divided into three two-hour periods: morning (9-11am), noon (12-2 pm) and afternoon (3-5pm), each of which had 1,440 images (240 images for 6 pools). A machine learning image processing tool (Tensorflow, www.tensorflow.org) was used to identify any fish in images, and then to identify the colors of elastomer marks on the fish. The workflow for image processing involved isolating all portions of images identified as fish, and then putative fish images with any identified elastomer marks. The software outputs an html file (in random order without identifying trial, time or location data) with all fish images with identified marks. This file was manually checked for accuracy and to correct any mistakes made by the AI. To do this, each image outputted by the software was scanned for two sets of criteria: (a) the presence of a fish in the image and (b) the color of the fish's mark. If the information given by the software was correct, the information was left as is. However, if the information was incorrect, it was manually corrected within the software. Processing images in this way, a single trial (4,320 images from all three time periods) could be processed by the AI with about 30 minutes of compute time followed by 1-2 hours of manually examining AI accuracy.

From the data composed by the software, pivot tables were made for each day a trial was conducted. The pivot tables were composed with the color in the rows, the color change in the columns, and the count of colors in the values. The pivot tables allowed for the compilation of false positives and negatives for each color, wrong color identifications (where the color identified by the software was corrected to another color manually), and total correct identifications for each trial day. Each of these categories (excluding total correct identifications) were summed for each color, and the average of each was taken. This showed the rate at which specific colors were identified as a false positive or false negative, and the rate at which a certain color was changed to another color.

The resulting data consisted of the number of verified counts of each species (male and female) in each pool at 30 second intervals. These counts were pooled into upstream (three pools) and downstream (three pools) mesocosm sections for each two-hour period. To summarize the distribution of fish, the distribution index (DI) was calculated representing the proportion of each species and sex found in upstream vs. downstream areas over that one hour ((total downstream – total upstream)/(total downstream + total upstream)). The mean distribution index (DI) shows the proportion of each species and sex found in the upstream and downstream areas of the homogeneous and heterogeneous treatment levels, averaged over all two-hour periods. For example, if a trial resulted in 90 *F. notatus* downstream and 30 upstream, the DI would be 0.5 ((90-30)/(90+30)). The DI is normalized so that a value of -1.0 indicates all individuals occupying a downstream position and 1.0 indicating all individuals upstream. I was also able to calculate the mean rate of coexistence (C), which is the proportion of pools that had the two species coexisting over a two-hour window.

The null expectation was a random distribution of species and sexes across all six pools in each treatment level, which would result in mean DI values not significantly different from 0. A mixed model repeated measures analysis of variance (lme4 R package) was used to test for differences in arc-sin transformed DI by species, sex, and treatment level with time as a random nested effect. After reviewing the overall model results, we used the same approach to test for species and sex differences in DI within each of the treatment levels.

CHAPTER III: RESULTS

Distribution Index

Male *F. notatus* had a strong preference for the upstream orientation (DI = 0.95) in the heterogeneous treatment but no preference in the homogeneous treatment (DI = 0). Female *F. notatus* had a similar pattern to its male counterpart, but not as strong (heterogeneous DI = 8.5, homogeneous DI = 4.5). Male and female *F. olivaceus* were not as selective as *F. notatus*, with males showing a slight preference for upstream (heterogeneous DI = 0.5, homogeneous DI = 0.1) and females actually preferring downstream habitats (heterogeneous DI = -1.5, homogeneous DI = 2.5). The differences between species were significant (species x treatment interaction, F = 32.3, P < 0.001). Overall, the pattern is consistent with *F. notatus* being more of a habitat specialist than *F. olivaceus* under these conditions. These habitat preferences match the distribution of the species observed in the Tombigbee basin (the source of fish for trials), which is the opposite of the usual habitat use of these species.

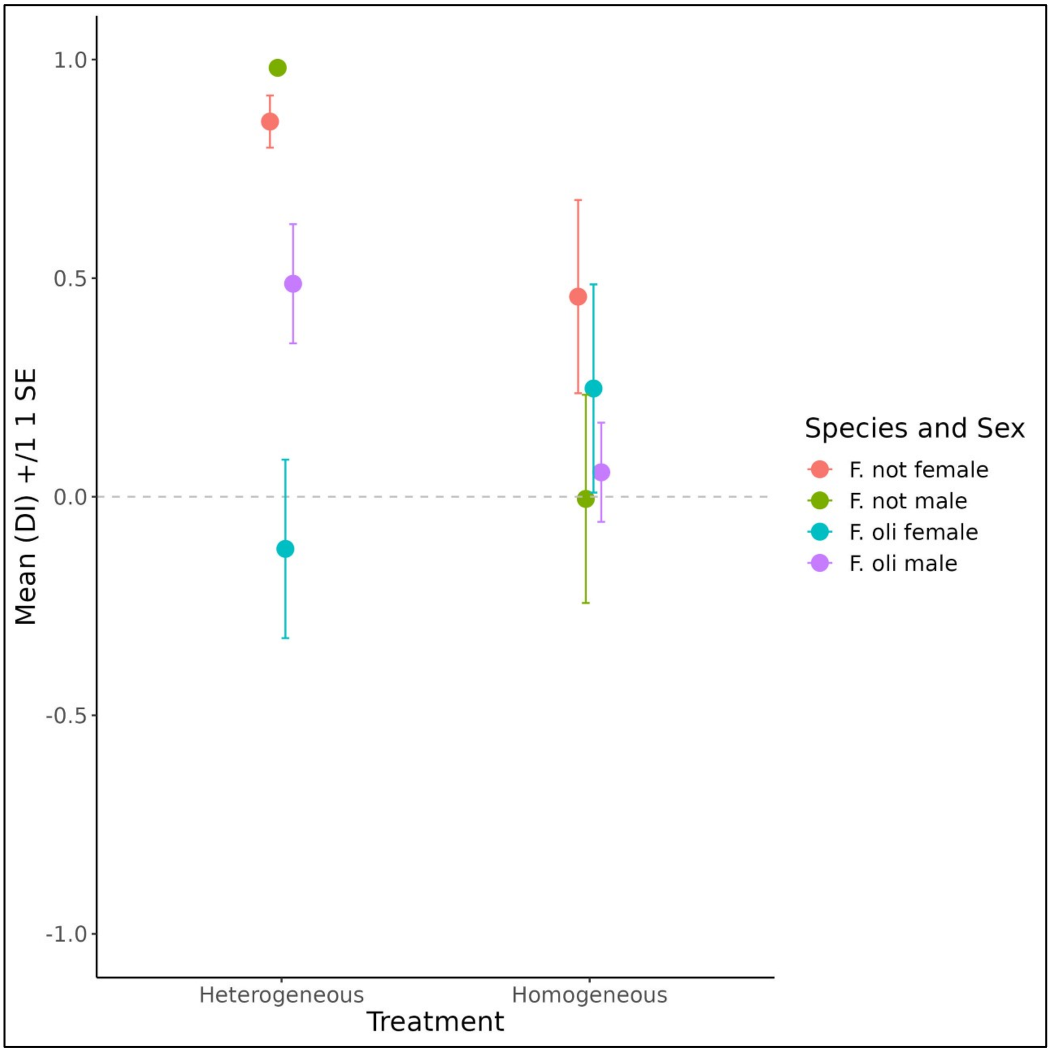


Figure 1: Distribution Index of Sexes and Species in Both Treatments. The distribution index of males and females in both *F. notatus* and *F. olivaceus* for heterogeneous and homogeneous treatment levels is shown. The DI is normalized to 1.0 indicating an upstream occupancy and -1.0 indicating a downstream occupancy.

Distribution Index ANOVA

	Mean sq	**NumDF**	**DenDF**	**F value**	**Pr (>F)**
species	6.8	1	83.1	68.9	P > 0.001*
treatment	0.8	1	7.9	7.6	0.02546*
sex	0.2	1	81.6	2.3	0.1
species:treatment	4.1	1	83.9	41.0	P > 0.001*
species:sex	0.6	1	82.9	5.6	0.02*
treatment:sex	3.8	1	82.0	37.9	P > 0.001*
species:treatment:sex	0.005	1	83.6	0.1	0.8

Table 1: Distribution Index ANOVA. Distribution index ANOVA results for species, treatment, and sex factors and their interactions between one another.

Coexistence

The mean rate of coexistence (Fig. 2) is the proportion of pools that had the two species coexisting over a two-hour window (i.e., a value of 1.0 indicates all pools had both species present at some time over every two hour window). Here, coexistence is defined as a ratio of the two species being more than 2/10 over a two-hour period, or both species accounting for more than 20% of the population. Using this definition, an example population would not be classified as coexisting if there were 3 *F. notatus* and 17 *F. olivaceus* occupying a pool over a two-hour window, as this ratio would be lower than 2/10. Alternatively, a population would be considered coexisting if there were 6 *F. notatus* and 14 *F. olivaceus,* since this ratio is higher than 2/10. The heterogeneous treatment level yielded a lower coexistence and standard error (C = 0.361, SE = 0.031) than the homogeneous treatment (C = 0.528, SE = 0.054). The coexistence in the two treatment levels was significant (Table 2). This decrease in coexistence for heterogeneous habitats is an indicator of potential habitat mediated reproductive isolation.

Coexistence ANOVA

	Df	**Sum Sq**	**Mean Sq**	**F-value**	**Pr (>F)**
Coexistence	1	0.20	0.20	8.34	0.007392
Residuals	28	0.67	0.02	-	-

Table 2: Coexistence ANOVA. Coexistence between *F. notatus* and *F. olivaceus* in the homogeneous and heterogeneous treatment levels.

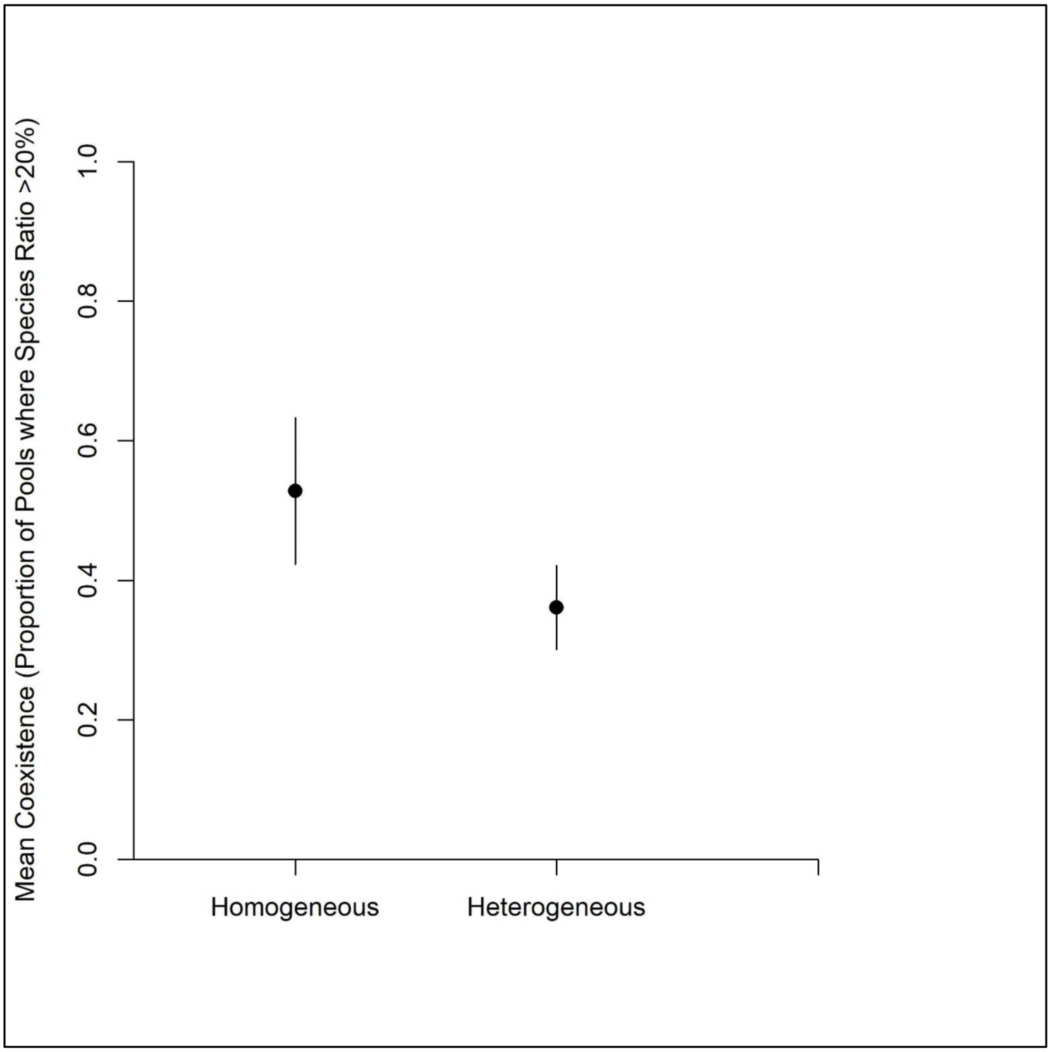


Figure 2: Mean Rate of Coexistence in Both Treatments. The mean rate of coexistence between *F. notatus* and *F. olivaceus* in homogeneous and heterogeneous treatments.

Artificial Intelligence Accuracy

The AI produced output that fell into several categories, including false positives, false negatives, wrong color, and correct identification. A "false positive" correction indicated a fish was absent in the image but was incorrectly identified as being present by the system. A "false negative" correction indicated a fish was present in the image but was overlooked by the AI system. A "wrong color" correction indicated a fish was correctly identified as being in the image, but the color of the elastomer mark on the fish was incorrectly identified. A correct sorting indicated the AI system accurately identified a fish and its elastomer mark color in the image. Overall, 14,475 pictures were taken by the system. After corrections, the AI system had an overall accuracy of 74.11%, with 21.59% false positives, 3.72% false negatives, and 0.58% wrong color identifications. The most common color associated with false positives and negatives was pink (37.15% and 39.89%, respectively). An example of an image is shown in Figure 3, where Figure 3A shows an image without any fish present and Figure 3B shows a correct identification of a fish and its mark. Figure 3B also shows a false identification of a fish, but is not considered a false positive since an elastomer tag color was not assigned.

Tag color	**False Positives**	**False Negatives**
Orange	31.8%	16.3%
Pink	37.2%	39.9%
Red	19.2%	19.3%
Yellow	11.9%	24.5%

Table 3: Artificial Intelligence Accuracy. False positives and negatives are shown for each elastomer tag color, with orange being the color with the highest percentage of false positives and pink with the highest percentage of false negatives.

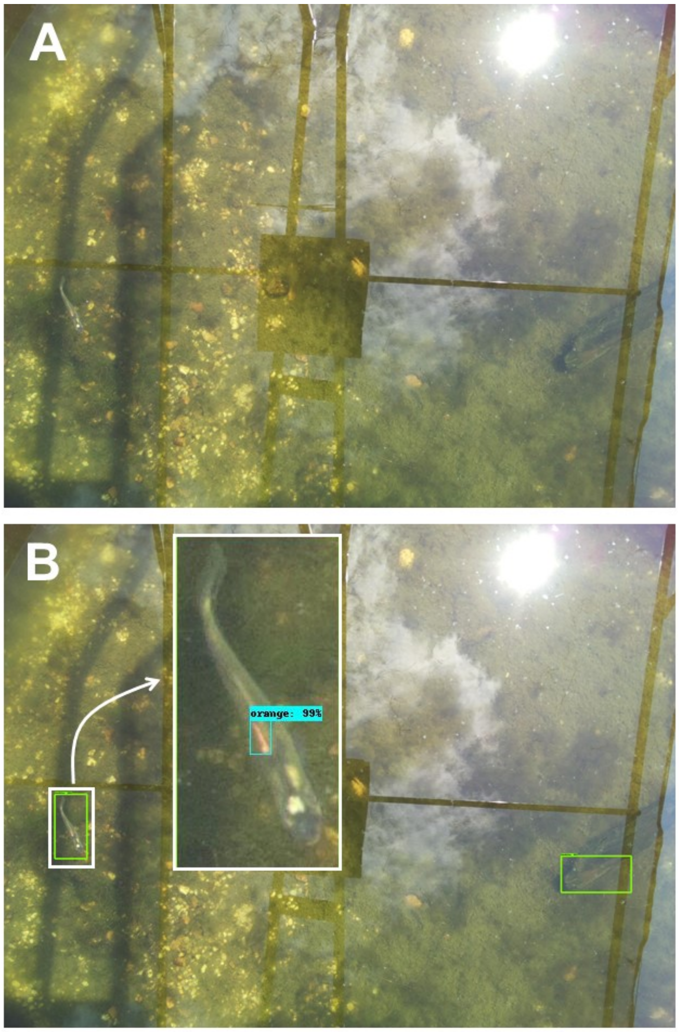


Figure 3(A/B): Artifical Intelligence Example Image. An image taken without any fish present or identified (Fig. 3A, top) and an image with a fish and its elastomer tag color correctly identified (Fig. 3B, bottom).

CHAPTER IV: DISCUSSION

Both species showed a preference for different habitats in the heterogeneous treatment. Topminnows for these trials were taken from a drainage where the two species displayed a reversed habitat preference (*F. notatus* residing upstream and *F. olivaceus* downstream). The hypothesis that both species would display a preference for the same upstream or downstream orientation was supported, as the observed orientation in trials matched their position in the field. Consequently, the null hypothesis of neither species showing any preference was rejected, as was the hypothesis that species would show preference for the typical habitat preference (*F. olivaceus* upstream, *F. notatus* downstream). This result suggests that neutral metapopulation processes such as mass effects are likely not an explanation for the reversed orientation distributions. Instead, local populations appear to have fundamentally different habitat preferences.

The fact that both species showed a preference in the heterogeneous habitat more than the homogeneous habitat is indicative of a preference for one habitat over another, and in these trials that includes four variables that were modified as part of the heterogeneous treatment level (i.e., depth, current velocity, substrate size, or canopy cover). It seems likely that the two species would isolate to a larger extent (larger DI difference, less coexistence) if more aspects of stream gradients were modified as part of trials. This study does not address how much habitat preference is derived from individual variables that differ between typical headwater and downstream habitats (Vannote et al., 1980).

Though both species exhibited stronger preferences in the heterogeneous treatment, DI values showed *F. notatus'* preference was much stronger than that of *F. olivaceus*. As this suggests *F. olivaceus* is more of a habitat generalist compared to *F. notatus*, the preference reversal observed in the Tombigbee River by both species is a more profound discovery for *F. notatus* than *F. olivaceus*. It should be noted that throughout the distribution, the two species do not generally coexist (Schaefer et al. 2016), possibly due to competitive interactions. A possible explanation may be that the Tombigbee possesses a more hospitable *F. notatus* habitat upstream compared to downstream and, as a result, *F. notatus* migrated upstream, which then forced *F. olivaceus* (as a generalist competitor) downstream. The headwaters of the Tombigbee where this reversal occurs have a unique geology with hard clay substrate instead of gravel and sand more typically found throughout the rest of the range (Duvernell & Schaefer, 2013). This could lend an explanation as to why *F. olivaceus*, being a habitat generalist, does not show as strong of a preference as *F. notatus* does for either orientation. However, this suggestion does not provide an explanation for the same reversals shown by these two species in other independent drainages. For example, *F. notatus* is found in the headwaters of the Glover River system in Oklahoma that is geologically similar to Ozark systems (Schaefer et al., 2016). Moreover, the upstream shift exhibited by both species during trials could also be explained by downstream pools being unwelcoming or inhospitable compared to the upstream pools.

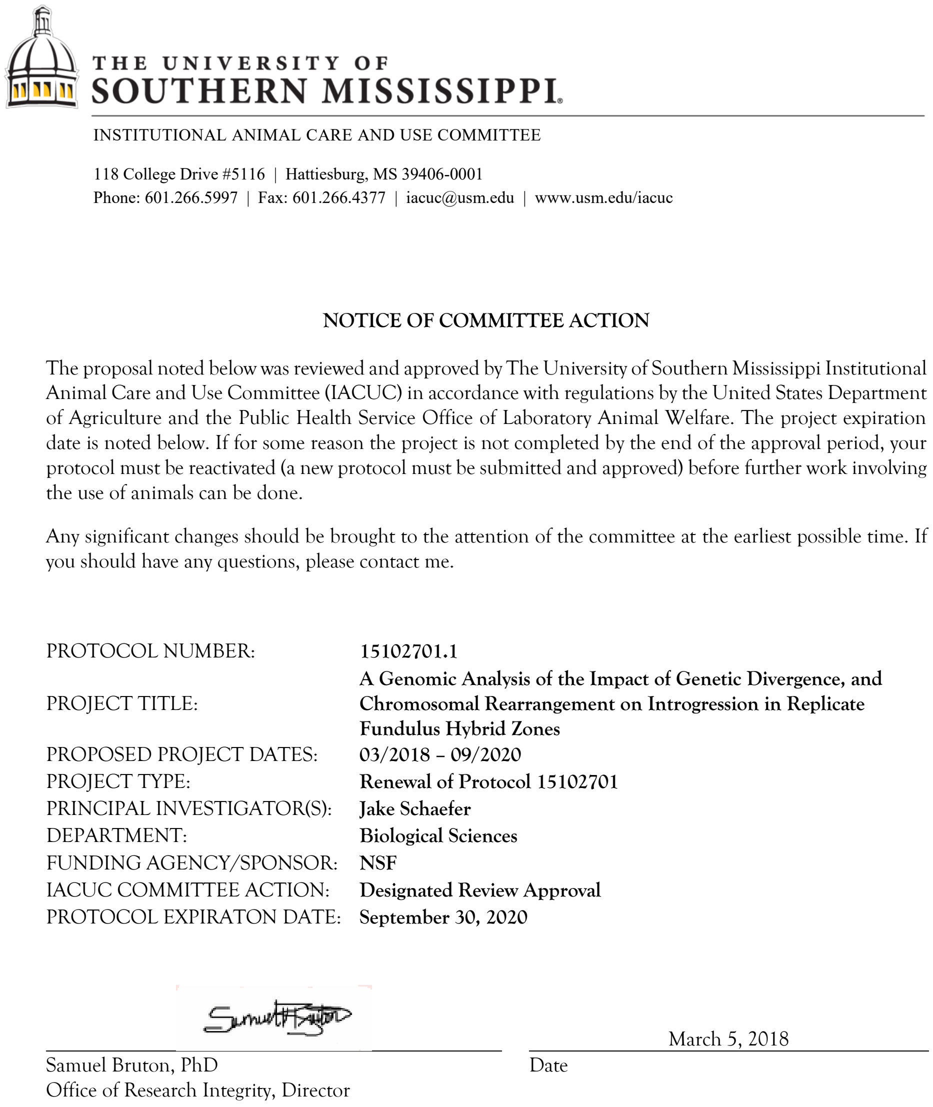
F. notatus and *F. olivaceus* are known to hybridize in confluences and sudden shifts in habitat (Schaefer et al., 2016). The coexistence data suggests the more distinct a habitat becomes, the less coexistence occurs between the two species, which would lower hybridization rates and possibly lead to reproductive isolation over time. When this understanding is applied to a broader spectrum of habitats and species, the implications of anthropogenic disturbances of habitats (eroding natural stream habitat gradients) could negatively impact resident populations (Duvernell & Schaefer, 2013). In particular, we would expect human disturbances that destroy native foliage, reduce canopy cover, alter hydrology, or homogenize habitats to contribute to the loss of distinct habitats, which would, in turn, increase coexistence of closely related species or lead to competitive exclusion (Levin, 1970). We would expect the former to likely result in increased hybridization, while the latter would possibly result in species shifting to new habitats. As a result of these consequences, environmental pressures of new habitats may cause evolutionary changes to a species, which would possibly result in reproductive isolation of two once closely related species.

The data collected do not address the relative roles of innate vs. acquired mechanisms in habitat preference. Habitat preference is clearly complex and encompasses a variety of factors that likely vary among species. As a result, pinpointing the drivers of a species' habitat preference is a difficult task to undertake. Innate and acquired habitat preferences come into play, often together, in niche selection (Takahashi & Masuda, 2019). Due to these species being sampled from a reversed hybrid zone and not being initially reared in a controlled environment, it is possible that the species' habitat selection was an acquired decision and not solely an innate one. This could only really be addressed by repeating this experiment and rearing both *F. notatus* and *F. olivaceus* in a controlled environment before conducting trials.

Artificial intelligence (AI) tools are increasingly common in a variety of disciplines and offer the potential to streamline data collection or processing. In this research, AI was used to process large numbers of photos (over 4,000 per trial) to identify the presence, species, and sex of a fish in trials. The accelerated data processing made this study possible, as other methods of tracking fish in these systems are more expensive or involve invasive procedures (surgical implanting of PIT tags) that are more likely to have negative effects on fish. Manually processing images would have reduced the size of the dataset by orders of magnitude. However, AI systems are not perfect, and errors were seen in misidentifying miscellaneous objects, riffles, or reflections as fish or assigning the wrong color to an elastomer tag. These errors were generally infrequent, and making manual corrections was still far more efficient than any other method of data collection. There still remain some constraints on the use of AI in this system such as water clarity, poor weather conditions (glare or light scattering on overcast days), and human error involved in the final data correction step. Overall, the AI performed well and streamlined collecting a large amount of data that would not have been possible without extensive costs or time investments.

The niche reversals seen in *F. notatus* and *F. olivaceus* may provide insight into evolutionary processes involved in niche modifications and selection. This research, in particular, gives insight to the resiliency of habitat preference on individuals taken from an area where the reversed preference is displayed and the potency of select environmental factors on habitat preference. In the trials, both species maintained their original habitat preferences, although *F. notatus*' preference was stronger than that of *F. olivaceus*. Following up this research by repeating trials with both species collected from a "flipped" orientation and an "expected" orientation, and then rearing them in a common garden would be beneficial in exploring innate versus acquired factors driving habitat preference. In addition, repeating trials with altered environmental conditions could also further our understanding of habitat preference of *F. notatus* and *F. olivaceus*. There is still much unknown of the inner workings of habitat preference, but research such as this provides pieces to the overall puzzle and helps to further our understanding by gaining a greater picture of fields such as evolution and ecology.

APPENDIX A: IACUC APPROVAL LETTER



THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

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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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