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Effects of Temperature Gradient, Substrate Composition, and Canopy Cover On the Spatial Distribution of Topminnow Species: *Fundulus notatus* and *Fundulus olivaceus*

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The University of Southern Mississippi

Effects of temperature gradient, substrate composition and canopy cover on the spatial distribution of two topminnow species: *Fundulus notatus* and *Fundulus olivaceus*

by

Austin King

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Approved by

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Abstract

Hybrid zones are locations where two interbreeding species coexist and hybridize. The spatial distribution of ecologically similar species is of primary interest in understanding the formation and stability of hybrid zones. These hybrid zones are of significance as they allow for insight into how speciation occurs naturally within the environment. A variety of factors may play a role in determining the spatial distributions of species within hybrid zones. Examples of these factors include variations in temperature gradients, substrate composition, and changes in canopy cover. *Fundulus olivaceus* and *Fundulus notatus* are generally found within upstream and downstream habitats respectfully. These habitats change in predictable ways in accordance to the river continuum concept. The purpose of this experiment was to test whether these environmental gradients influence distribution and the structure of hybrid zones. For this study, roughly 40 specimens of each sex and species were collected for a total of 160 fishes. Each specimen was marked with an elastomer tag that coded for species and sex. Three mesocosm treatments were created; a control, heterogeneous and temperature only. The control had no change in habitat variables throughout the treatment while the heterogeneous treatment was arranged to mimic a stream habitat in accordance to the river continuum concept. This includes a shallower, faster moving, colder upstream and a deeper, slower moving, warmer downstream. The homogeneous treatment was arranged identical to the control with only a temperature gradient present. Ten fish of both sex and species, 40 total, were placed in each treatment. It was found that both species exhibited habitat preference when presented with changes in habitat structure. Of the two species, *F. notatus*, males in particular, exhibited the highest mean index of upstream vs downstream bias.

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Introduction

Hybrid zones have long been of interest to ecologists and evolutionary biologists because they allow one to study naturally occurring populations where speciation is incomplete (Hewitt 1988). Hybrid zones are formed when two ecologically similar species coexist and reproduce. It is not clear what role environmental gradients play in creating or stabilizing naturally occurring hybrid zones. More broadly, the role that spatial distribution has on a local species has widely been a topic of interest to ecologists. Being able to understand the forces that shape species distributions will allow ecologists to understand shifts in distributions and the underlying forces that form hybrid zones.

Stream ecosystems are ideal places to study the role of environmental gradients in determining species distributions and coexistence as many of the most important variables change in linear and predictable ways. These changes are described by the River Continuum Concept (RCC: Vannote et al. 1980). The RCC describes linear and predictable changes in temperature, substrate, canopy cover, productivity, and hydrology as one progress from small headwater streams to larger rivers (Giakoumi and Kokkoris, 2012). Headwater streams tend to be narrower, shallower, and cooler, with larger substrate and faster flow rates. Progressing downstream, streams increase in depth, decrease in flow rate (due in part to reduce stream bed slope), and widen giving way to more direct sunlight which increases temperature and productivity. Erosional processes push smaller substrate downstream producing a sediment gradient with larger substrate dominating upstream and silt and sand dominating in larger rivers. Within stream networks, confluences represent abrupt changes in the RCC, and places where disparate habitats may be spatially close (e.g. a small headwater stream meeting a larger river).

One of the primary factors that influences habitat selection, and thus the spatial distribution, of fish is water temperature (Magnusson et al., 1979, Plumb and Blanchfield, 2008). As aquatic ectotherms, environmental temperature is directly correlated with metabolic rate and a number of related physiological properties. In laboratory trials, it is well established that fish will select habitats based, in part, on a thermal optima where they can maximize their fitness and growth (Bostrom, *et. al.* 2010). It is not as clear how temperature influences habitat selection in a field setting where there is extensive diel, seasonal, and microhabitat variation in temperature.

A second possible factor that is demonstrated to affect a species' spatial distribution is substrate (Luckhurst & Luckhurst, 1978). Substrate composition is often cited as one of the most important determinants of fish community composition (Szedlmayer and Howe, 1997). A variety of anthropogenic disturbances to watersheds alters sediment dynamics by increasing bank erosion and the input of finer sediments. These fine sediments fill interstitial spaces in larger substrates that are often home to prey, or necessary for early life history stages of some species. Specific substrates may also be necessary for the presence of macrophytes. For some fish species, these may be imperative for egg deposition sites. Thus, substrate structure plays a vital role in fish distribution (Juanes, 2007, Boussu, 1954; Eklo¨v and Greenberg, 1998).

Whether it is macrophytes within the littoral zone or vegetation within the riparian zone, canopy cover decreases along the river continuum. With this change in canopy cover, comes a change in productivity and the input of terrestrial materials (Platts and Nelson, 1989). The increased shade upstream greatly reduces primary productivity but provides additional allochthonous material in the form of leaf litter and terrestrial insects. For many headwater species of fish, terrestrial insects are the primary diet item. As you move downstream, streams get proportionately less allochthonous input and instead are fueled by primary productivity

(Smokorowski and Pratt, 2006). Fish living downstream are therefore typically less specialized for feeding on small invertebrates and more often piscivores, planktivores or detritivores.

As a hyponeustonic family of fish, the family Fundulidae, also known as the topminnows, are a group of organisms that primarily live just beneath the surface of the water. These fish can be found in both fresh and brackish ecosystems (Ross, 2001). Terminally oblique mouths with projectile jaws, and poorly developed or incomplete lateral lines are but a few of the notable characteristics of this topminnow family (Wiley, 1986). The family consists of three genera (*Fundulus*, *Lucania*, and *Leptolucania*) with a total of 40 named species. Fundulids have a wide range of feeding preferences with the majority feeding from the surface and fewer consuming benthic organisms or macrophytes (Ross, 2001). Being found in both freshwater and brackish, the range of the family Fundulidae is broad.

The black-stripe topminnow (*F. notatus*) and black-spotted topminnow (*F. olivaceus*) are two very closely related species that can be found together in many drainages throughout their broad distribution. Both species' native ranges overlap significantly. *Fundulus notatus* can generally be found from the southern Great Lakes tributaries, Mississippi River Basin, and other Gulf Coastal drainages from Tombigbee River portion of Mobile Basin west to San Antonio Bay, Texas (Etnier and Starnes, 1993). The range for *F. olivaceus* is smaller although they are still widely abundant within the central and lower Mississippi River Basin, and Gulf Coastal drainages from Choctawhatchee River, Florida, through San Jacinto River, Texas (Etnier & Starnes, 1993). Throughout most of its distribution, *F. notatus* is found downstream in the backwaters which consist of a low-gradient slower moving water while *F. olivaceus* is usually found within moderately high-gradient headwater streams (Braasch and Smith, 1965; Thomerson, 1966; Thomerson and Woolridge, 1970; Howell and Black, 1981). In drainages where both *F. notatus* and *F. olivaceus* occur, coexistence and hybridization is primarily near confluences. Hybrid zones are typically narrow and limited to a few river kilometers up or downstream of confluences. This is important as confluences represent breaks within the RCC where you get rather abrupt changes in habitat over a small space. Interestingly, when either species is found alone in a drainage it will occupy all habitats from headwaters downstream (Schaefer *et al*., 2009; Schaefer *et al*., 2011a). These two species therefore represent an ideal system in which to ask basic questions about what determines species distributions along ecological gradients (Schaefer *et al*., 2009; Schaefer *et al*., 2011a).

The purpose of this study was to better understand what might contribute to the observed distribution of *F. notatus* (downstream) and *F. olivaceus* (upstream) with coexistence and hybridization centered around confluences. I tested the hypothesis that the species would segregate along an artificial stream gradient within an experimental setting. When the species responded to gradients in an experimental setting, that allowed for manipulation of these gradients (e.g. removing one factor at a time) to see which was most important in determining distribution. In a second treatment, I asked if the species would segregate along a gradient that featured only a temperature difference.

Methods and Materials

Fish Collections

Study specimens were collected by two methods; seining and dip-netting. These collections were conducted in locations known to possess high abundance of each species. The Bouie River located near Highway 59 bridge was the primary collection site for *F. olivaceus* while the Pascagoula River and its drainages within the Pascagoula Wildlife Management area was the primary collection site for *F. notatus*. Field collections continued until roughly 40 specimens of each sex and species were collected for a total of 160 fish. Fish were transported to Lake Thoreau Environmental Center where they were housed within a holding tank to allow for acclimation for two days. After acclimation, each fish was then individually anesthetized using tricaine methanesulfonate (MS222) and injected with an elastomer tag dependent on the specimens' species and sex. Regarding species differentiation, *F. olivaceus* was marked with an orange elastomer tag while *F. notatus* was injected with a green tag. In respect to the sex of each species, the tag was placed immediately behind the skull for females and immediately before the caudle fin for males.

Mesocosms

Experiments were conducted in stream mesocosms (Matthews et al. 2006) at the Lake Thoreau Environmental Center. The mesocosm setup was comprised of six circular shaped tanks 183 cm in diameter. Each tank was connected by a rectangular 'riffle' that is 43 cm wide and 183 cm long. These riffles allowed constant flow of water throughout the system and allowed the fish to disperse among the pools. Conditions within the three mesocosms were modified into three treatments (hereafter control, heterogeneous, and temperature only). Within each mesocosm, three tanks were designated headwater and three downstream. The control mesocosm was homogeneous with sand and gravel as the sediment, no canopy cover, uniform depth of over 30 cm in pools, negligible flow and ambient temperature. The heterogeneous mesocosm was modified to have different habitats up and downstream to mimic a natural stream gradient. Within the headwater portion, the sediment was composed of cobble and gravel in the riffles and gravel and sand in the

Figure 1. Picture of upstream portion of heterogeneous mesocosm treatment with larger substrate, shallow water, increased canopy cover and current velocity.

pools (Fig. 1). Downstream sediment composition changed to sand and gravel in pools and riffles. Canopy cover was increased in the headwaters by installing camouflage netting four feet above the water-surface. Pumps and chillers were installed to increase the flow and decrease temperature (1º C below ambient) in the headwater section. Downstream, submerged heaters increased the temperature 1º C above ambient. Finally, the upstream segment had additional substrate to yield a uniform shallow (<20 cm) depth. The result was that the heterogeneous treatment had upstream conditions that were cooler, shallower, higher flow, and with larger substrate. The third treatment (temperature only) was similar to the control except that the temperature alone was modified as in the heterogeneous (Fig. 2).

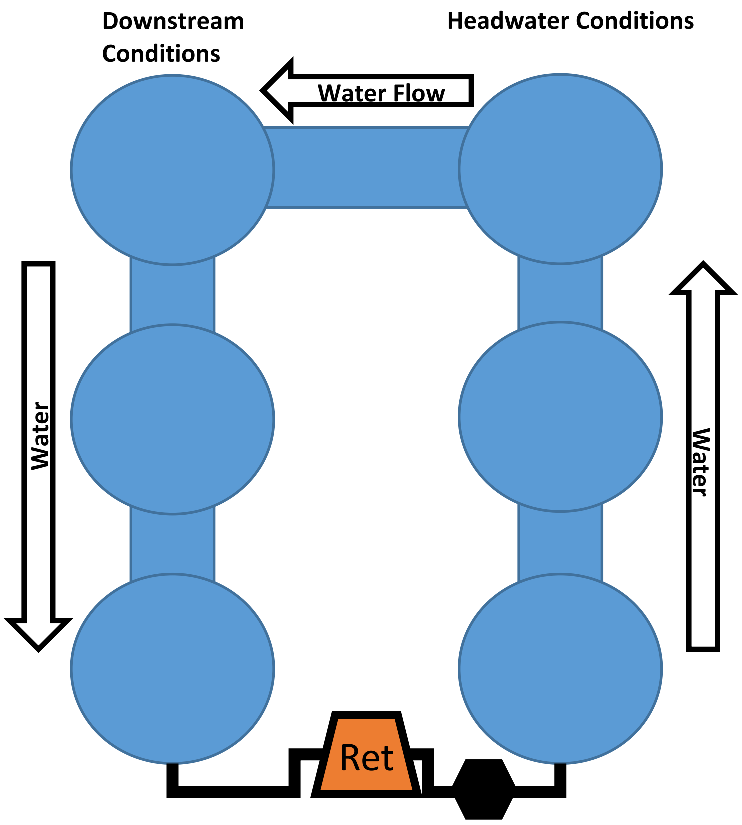


Figure 2. Diagram depicting a top view of one mesocosm unit. Pools (round) and riffles (rectangles) are each 183 cm in length and habitats within were modified to represent headwater (first three pools and riffles) and downstream (last three) habitats.

Ten fish of both sex and species, 40 total, were randomly distributed among each pool within each mesocosm. This gave way to a grand total of 120 fish throughout all three mesocosms. A 24-hour period was given to allow the fish to acclimate to the new environments. After acclimatization, one GoPro camera was placed over each tank. To avoid bias, the choice of which treatment to use during each observed time was chosen at random. The placement of the cameras during each observation, morning or afternoon, on either the control, heterogeneous or homogeneous treatments was also random to avoid systematic bias. These cameras took one picture every five minute for two hours. This happened twice daily between 9am-11am and again from 2pm-4pm for eight days completing one trial. A total of three trials (each with eight days of observation) were completed.

Data Analysis

After all pictures had been gathered and categorized properly, every photo was reviewed to observe and record the number of each species, according to sex, found within each pool. This allowed for a rough overview of pool preference, thus environmental (upstream vs. downstream) preference, found within each species and sex. From here, an index was calculated (difference between the number upstream and downstream divided by the total observed) to represent the proportion of each species and sex found in upstream vs. downstream locations. This index ranged in values from -1 (all individuals found downstream) to 1 (all individuals found upstream) and was calculated using the location data from all pictures at each five minute interval. The null expectation, if there is no preference for up or downstream habitat, is an index value of 0. Once all pictures had been reviewed, a repeated measures analysis of variance (ANOVA-Type III; lme4 package, R Development Core Team, 2009) was run on the data gathered to analyze the differences among index means by species, treatment and sex. For factors which were statistically significant, post hoc t-tests were run to test for individual differences between species by sex and treatment.

Results

Cameras recorded pictures for a total of 48 two-hour periods (15 heterogeneous, 16 control, and 17 temperature only). The unbalanced design was a result of some trials being discarded because weather conditions did not yield pictures for which I could reliably identify individual fish. This was usually due to rain disrupting the water surface or overcast conditions producing glare that blocked portions of the pools. There was a total of 34,560 photos. Due to time constraints, I analyzed every fifth photo (five minute intervals) to detect the presence of the tagged fish in each location. There was a total of 7,200 photos analyzed, 2,400 per trial, yielding a total of 3,016 individual fish observations (Fig. 3). The number of fish observed was consistent across treatments (control: 783, heterogeneous: 1027, and temperature only: 1206) and averaged 62.8 observations for each two-hour observation period. One unexpected trend was the disparity in the number of observations for males vs. females. Despite the sex ratios being equal in all treatments, there were 2032 observations of females and 984 observations of males. This is most likely a result of behavioral differences between the sexes as males tend to be more mobile than

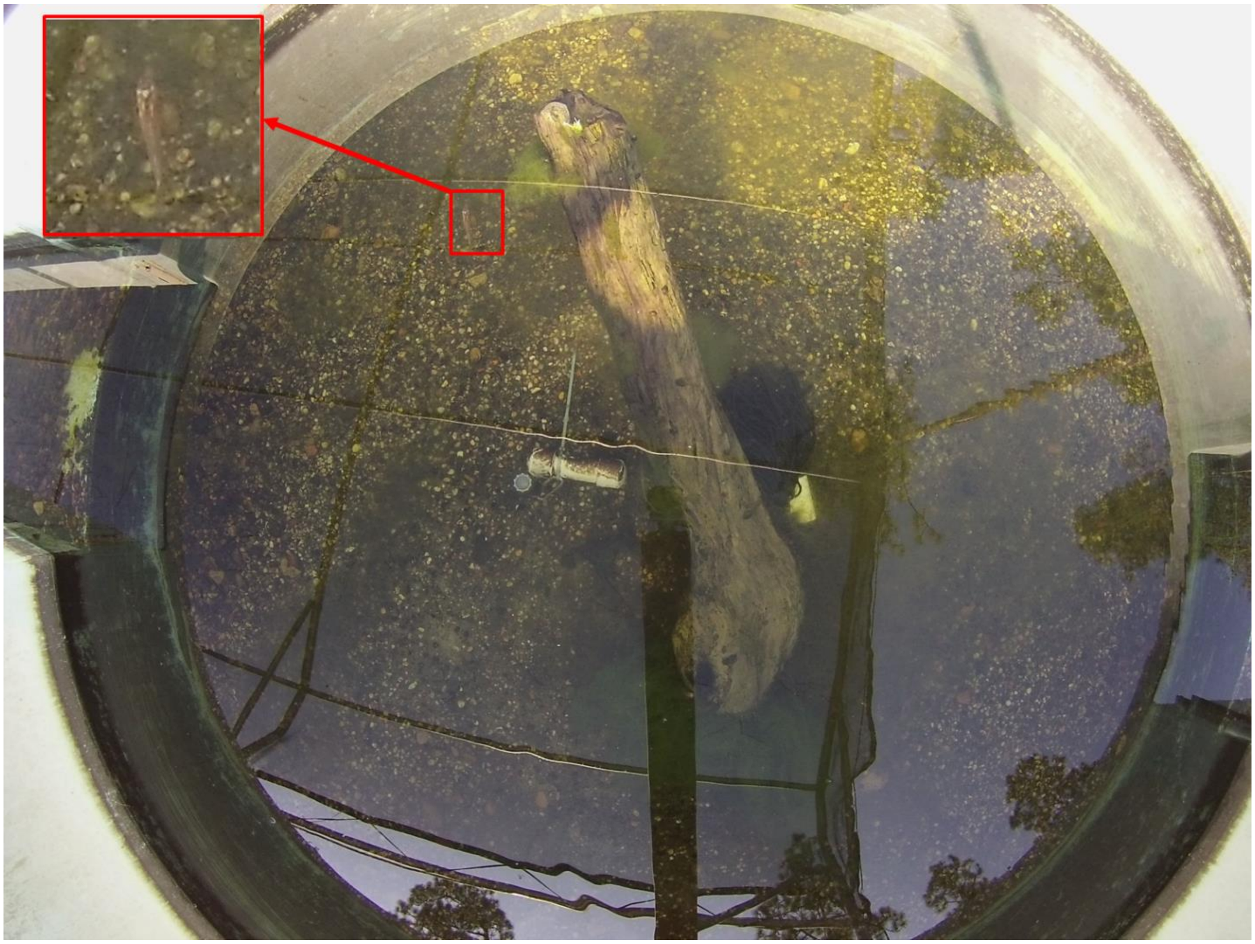


Figure 3. One of the 7,200 photos examined to identify the location of fish. A single individual is highlighted and zoomed to demonstrate the orange anterior elastomer mark identifying this individual as a female *F. olivaceus*.

females. Females were therefore more likely to be stationary and observed on multiple successive pictures. There was a significant difference in spatial distribution index among treatments (ANOVA, F=53.646, P<2.00E-16) and species x treatment (F=4.645, P<0.01017) interactions. There was no difference between the sexes or in any of the other interactions (Table 1). Of the seven tested interactions, two were discovered to be statistically significant (Table 1).

There were a total of 1513 *F. notatus* (975 females and 538 males) observed in the photos. With this data, six t-test were run to test for differences between both sex and treatment. The means and standard error gathered from these t-test were then formatted into a bar graph with error bars (Fig. 4). When comparing the means and standard error of each species by sex to that of the control there was variability as expected. Overall, F. notatus responded strongly to the experimental gradient with index scores in the control closer to 0 (averaged $\bar{x} = 0.1981 \pm 0.0716$) compared to the heterogeneous where most individuals were found in downstream habitat (averaged $\bar{x} = 0.5354 \pm 0.0595$). Male *F. notatus* seemed to have a stronger response than females, but these results were not significantly different (heterogeneous averaged $\bar{x} = 0.6333 \pm 0.0639$, temperature only averaged $\bar{x} = -0.2021 \pm 0.1186$, control averaged $\bar{x} = 0.2263 \pm 0.0944$).

There were a total of 1503 *F. olivaceus* (1057 females and 446) observed. *F. olivaceus* did not respond as strongly to the gradient as *F. notatus*. Female index values increased marginally from the control (averaged $\bar{x} = 0.1914 \pm 0.0826$) to heterogeneous (averaged $\bar{x} = 0.3247 \pm 0.0970$). Male F. olivaceus decreased in heterogeneous treatment (averaged $\bar{x} = 0.2475 \pm 0.0918$) when compared to the control (averaged $\bar{x} = 0.3052 \pm 0.1113$) (Fig. 5).

Table 1. Results from a repeated measures ANOVA testing for differences in spatial distribution between species, treatment, and sex. Statistical significance at P<0.05 is indicated with an asterix.

Interaction	Sum Sq.	Mean Sq.	*df*	*F*	*P*
Species	0.584	0.5844	1	1.189	0.27621
Treatment	52.732	26.3662	2	53.646	2.00E-16*****
Sex	0.79	0.7903	1	1.608	0.20556
Species x Treatment	4.566	2.283	2	4.645	0.01017*****
Species x Sex	0.283	0.2826	1	0.575	0.44874
Treatment x Sex	0.142	0.071	2	0.144	0.86558
Species x Treatment x Sex	0.919	0.4594	2	0.935	0.39361

Overall, the stronger response in *F. notatus* is what generated the significant interaction between species and treatment. Distributional patters for *F. olivaceus* did not seem to differ from the control while *F. notatus* showed a strong response to experimental gradients.

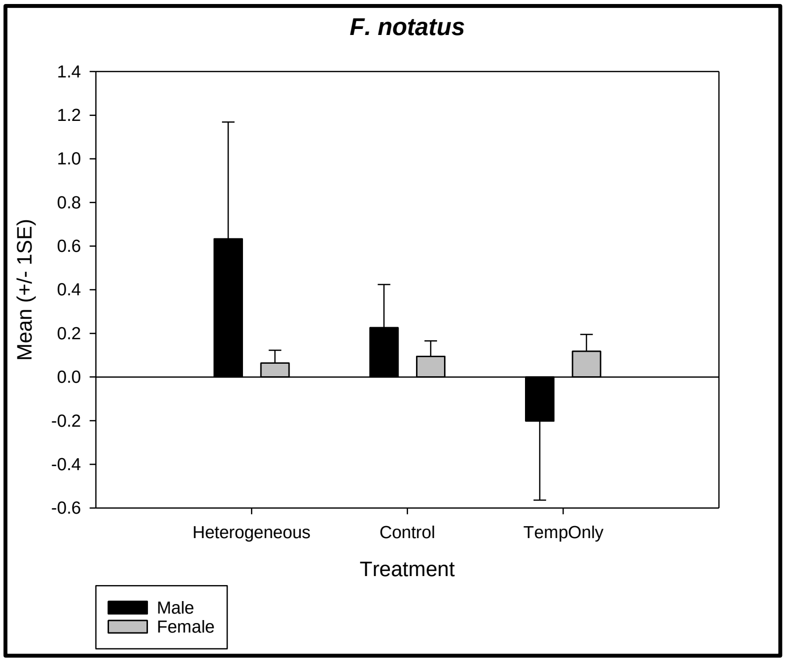


Figure 4. Mean and standard error index values for male and female *F. notatus* in the heterogeneous, control and temperature-only treatments. Index values of 0 indicate even distribution upstream and downstream. Negative and positive values indicate an upstream and downstream bias in distribution, respectively.

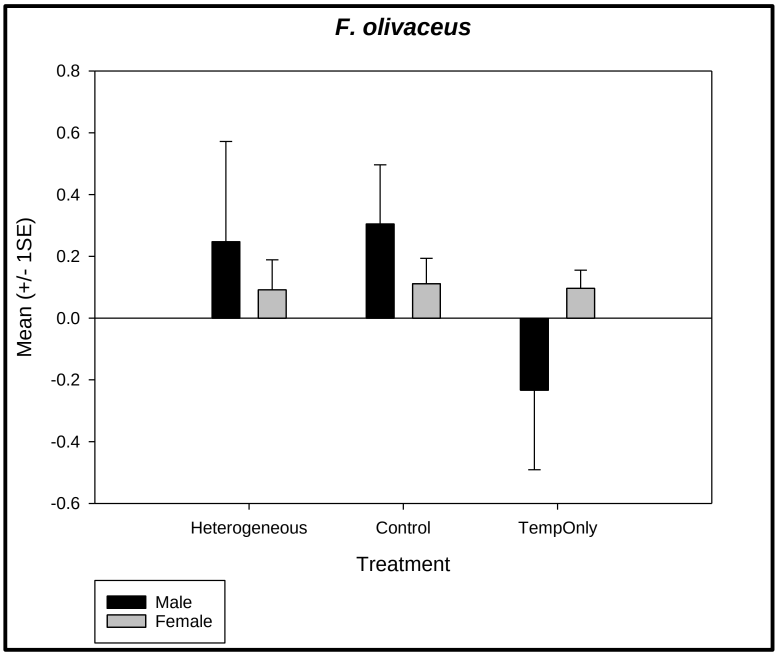


Figure 5. Mean and standard error index values for male and female *F. olivaceus* in the heterogeneous, control and temperature-only treatments. Index values of 0 indicate even distribution upstream and downstream. Negative and positive values indicate an upstream and downstream bias in distribution, respectively.

Discussion

In this study, it was predicted that both species would segregate along the river continuum gradients within an experimental setting. My predictions were only partially supported as just one of the two species did. Of the two species, *F. notatus* segregated along the gradient while *F. olivaceus* did not. This is consistent with observed distributional patterns within hybrid zones. While hybrid zones are centered around confluences, there is a consistent pattern of *F. olivaceus* distribution extending out of headwaters and into large river system. The opposite is not true, as *F. notatus* are rarely found in headwater streams or above hybrid zones. Both the field observations and results of this experiment suggest *F. olivaceus* is more of a habitat generalist than *F. notatus*.

Neither species responded significantly to the temperature only treatment meaning temperature alone is likely not responsible for observed distributional patterns and structure of hybrid zones. The temperature-only treatment differed most from the other two in that the index values were negative for both species, indicating that in those trials both species preferred the upstream segments that were cooler. Trials were conducted in the summer when stream temperatures were warm, and the cooler headwaters would have been closer to the estimated thermal optima of both species (Schaefer 2012). Conversely, it is not clear why there was a slight downstream bias in control and heterogeneous treatment. Overall, the mean ratio for the control was 0.23 with a 95% confidence interval from 0.15 to 0.31. Thus, fish did not distribute randomly in the control trial.

Of the two species, *F. notatus* exhibited the highest mean index of upstream vs downstream bias given a variable temperature gradient. Also, when compared to the control, *F. notatus* within the heterogeneous treatment observed the highest rate of possible bias (Fig. 2).

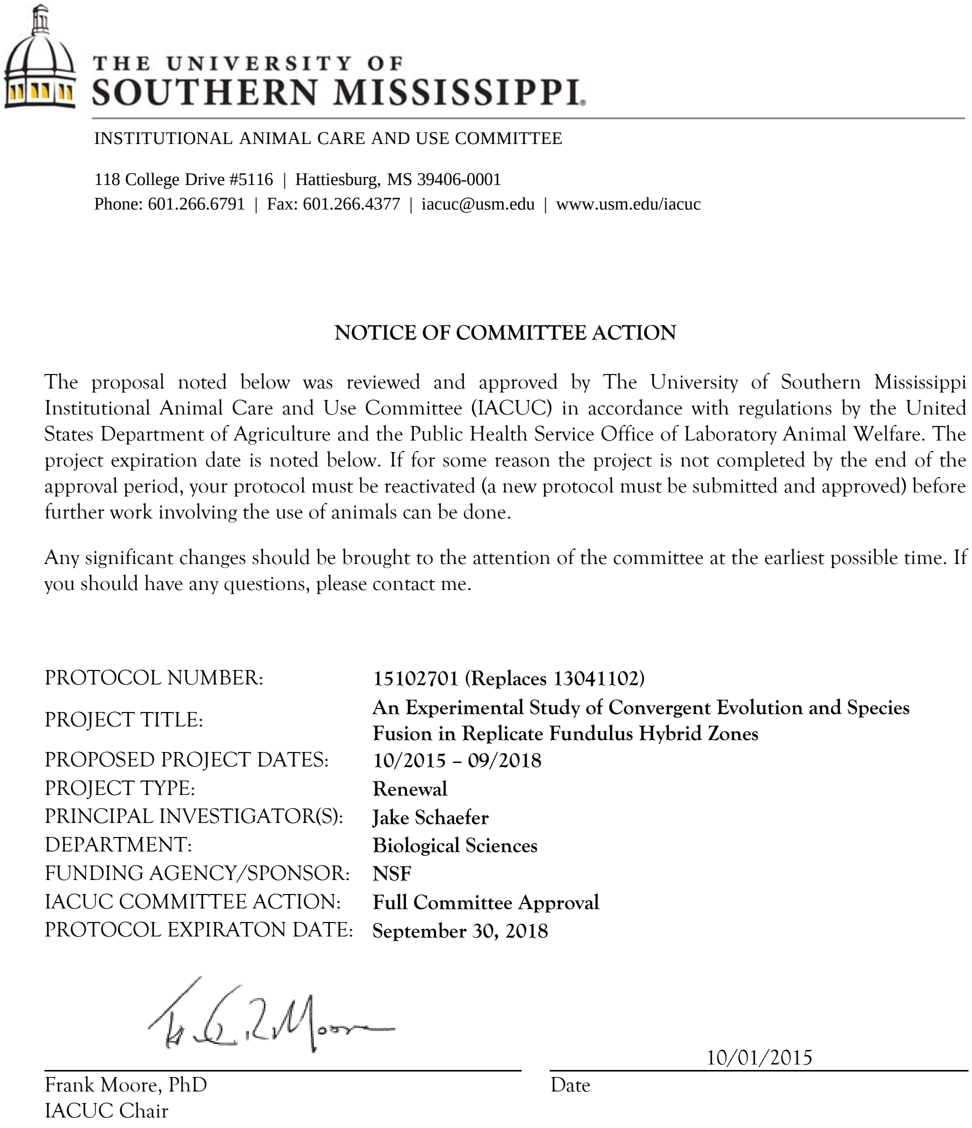
This could be attributed to *F. notatus*' greater performance breath and temperature tolerance over *F. olivaceus* within the presence of fluctuating temperatures (Schaefer, 2012). Having the ability to withstand a variety of temperature gradients would allow for a broad temperature based distribution and less needed variability within this distribution due to the loss of a selective pressure. With *F. notatus* primarily found downstream, they experience slightly different selection pressures than that of *F. olivaceus*. These include factors that coincide with the river continuum concept: increased water temperatures and slower water velocity. Being downstream, these factors can change drastically from reach to reach. According to Schaefer (2012), hatch success reaction norms suggest that *F. notatus* are more eurythermic than that of *F. olivaceus.* This would explain the increased variability seen within *F. notatus* as they are able to tolerate higher fluctuations in temperature.

With regards to *F. olivaceus*, it was found that they displayed a lesser amount of variance when presented with only a temperature gradient. Likewise, based on the t-test ran (Figure 3), *F. olivaceus* presented the lowest rate of variability within the heterogeneous treatment when compared to the control. Due to *F. olivaceus* primarily being found in upstream habitats, it has been hypothesized that their increased metabolic rates are adaptive to their colder, faster moving environment (Schaefer, 2012). According to Schaefer (2012), *F. olivaceus* observed low hatch success, increased developmental deformities and slower development at temperatures other than optima. These factors help infer that *F. olivaceus* is less eurythermic than *F. notatus*, thus limiting their spatial distribution.

The method of obtaining photos may have led to a sampling bias. Having the GoPro attached to a wooden arm allowed for full view of each tank. However, some photos had shadow areas around the edges of the tank limiting the field of view and possibly allowing for incorrect species count. Sun glare and shadows from artificial canopy cover also may have played a small role in possible sampling bias.

From the results obtained, it can be concluded that the ever-changing environmental variables along the river continuum plays an intricate role in the spatial distribution of both *F. notatus* and *F. olivaceus*. This strong relationship between species and environment sets the stage for hybrid zones being formed near confluences. This is not surprising since confluences are regions where two rivers meet thus allowing two different environments to converge. Of the treatments tested, temperature was the lowest selective pressure as it produced the lowest mean index of an upstream vs downstream bias seen within each species by sex. However, when added with other variables such as a substrate and canopy cover gradient, the mean index of an upstream vs. downstream bias greatly increased as habitat variation inevitability increased. Having the highest mean index in the face of a temperature gradient, it can be concluded that of the species tested, *F. notatus* has the highest tolerance to changes within the river continuum.

APPENDIX A
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE



THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.6791 | 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 (Replaces 13041102)**
PROJECT TITLE:	**An Experimental Study of Convergent Evolution and Species Fusion in Replicate Fundulus Hybrid Zones**
PROPOSED PROJECT DATES:	**10/2015 – 09/2018**
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Jake Schaefer**
DEPARTMENT:	**Biological Sciences**
FUNDING AGENCY/SPONSOR:	**NSF**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2018**

Frank Moore, PhD
IACUC Chair

10/01/2015
Date

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