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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

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirements for the Degree of
Bachelor of Science
in the Department of Biological Sciences

May 2017

Approved by

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Honors College

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 respectively. 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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1 **Introduction**

2 Hybrid zones have long been of interest to ecologists and evolutionary biologists because
3 they allow one to study naturally occurring populations where speciation is incomplete (Hewitt
4 1988). Hybrid zones are formed when two ecologically similar species coexist and reproduce. It
5 is not clear what role environmental gradients play in creating or stabilizing naturally occurring
6 hybrid zones. More broadly, the role that spatial distribution has on a local species has widely
7 been a topic of interest to ecologists. Being able to understand the forces that shape species
8 distributions will allow ecologists to understand shifts in distributions and the underlying forces
9 that form hybrid zones.

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

23

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

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

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

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

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

58 The black-stripe topminnow (*F. notatus*) and black-spotted topminnow (*F. olivaceus*) are
59 two very closely related species that can be found together in many drainages throughout their
60 broad distribution. Both species' native ranges overlap significantly. *Fundulus notatus* can
61 generally be found from the southern Great Lakes tributaries, Mississippi River Basin, and other
62 Gulf Coastal drainages from Tombigbee River portion of Mobile Basin west to San Antonio
63 Bay, Texas (Etnier and Starnes, 1993). The range for *F. olivaceus* is smaller although they are
64 still widely abundant within the central and lower Mississippi River Basin, and Gulf Coastal
65 drainages from Choctawhatchee River, Florida, through San Jacinto River, Texas (Etnier &
66 Starnes, 1993). Throughout most of its distribution, *F. notatus* is found downstream in the
67 backwaters which consist of a low-gradient slower moving water while *F. olivaceus* is usually
68 found within moderately high-gradient headwater streams (Braasch and Smith, 1965;
69 Thomerson, 1966; Thomerson and Woolridge, 1970; Howell and Black, 1981). In drainages

70 where both *F. notatus* and *F. olivaceus* occur, coexistence and hybridization is primarily near
71 confluences. Hybrid zones are typically narrow and limited to a few river kilometers up or
72 downstream of confluences. This is important as confluences represent breaks within the RCC
73 where you get rather abrupt changes in habitat over a small space. Interestingly, when either
74 species is found alone in a drainage it will occupy all habitats from headwaters downstream
75 (Schaefer *et al.*, 2009; Schaefer *et al.*, 2011a). These two species therefore represent an ideal
76 system in which to ask basic questions about what determines species distributions along
77 ecological gradients (Schaefer *et al.*, 2009; Schaefer *et al.*, 2011a).

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

86

87 **Methods and Materials**

88 *Fish Collections*

89 Study specimens were collected by two methods; seining and dip-netting. These
90 collections were conducted in locations known to possess high abundance of each species. The
91 Bouie River located near Highway 59 bridge was the primary collection site for *F. olivaceus*
92 while the Pascagoula River and its drainages within the Pascagoula Wildlife Management area

93 was the primary collection site for *F. notatus*. Field collections continued until roughly 40
94 specimens of each sex and species were collected for a total of 160 fish. Fish were transported to
95 Lake Thoreau Environmental Center where they were housed within a holding tank to allow for
96 acclimation for two days. After acclimation, each fish was then individually anesthetized using
97 tricaine methanesulfonate (MS222) and injected with an elastomer tag dependent on the
98 specimens' species and sex. Regarding species differentiation, *F. olivaceus* was marked with an
99 orange elastomer tag while *F. notatus* was injected with a green tag. In respect to the sex of each
100 species, the tag was placed immediately behind the skull for females and immediately before the
101 caudle fin for males.

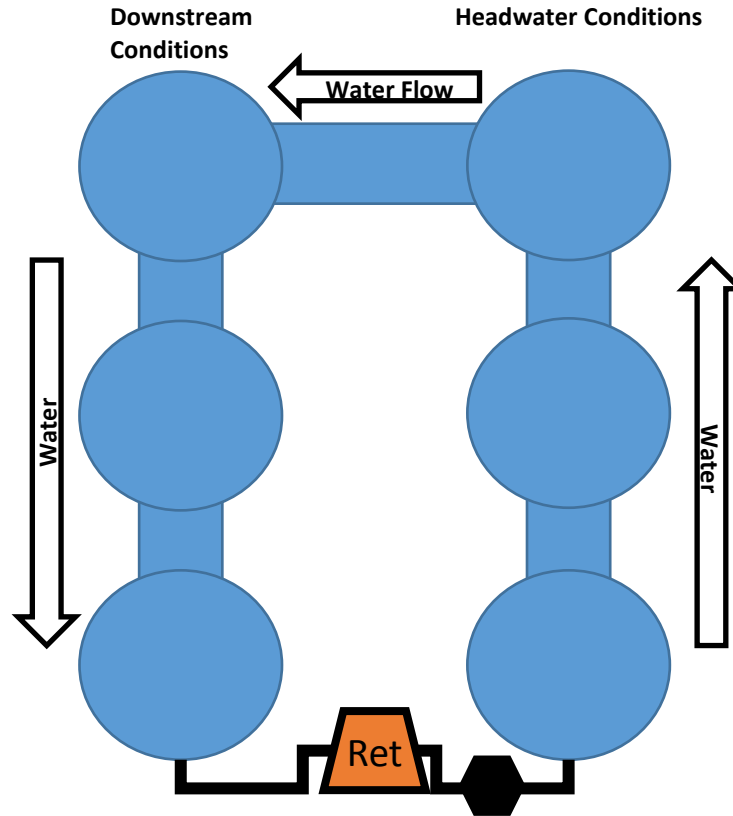
102 *Mesocosms*

103 Experiments were conducted in stream mesocosms (Matthews et al. 2006) at the Lake
104 Thoreau Environmental Center. The mesocosm setup was comprised of six circular shaped tanks
105 183 cm in diameter. Each tank was connected by a rectangular 'riffle' that is 43 cm wide and 183
106 cm long. These riffles allowed constant flow of water throughout the system and allowed the fish
107 to disperse among the pools. Conditions within the three mesocosms were modified into three
108 treatments (hereafter control, heterogeneous, and temperature only). Within each mesocosm,
109 three tanks were designated headwater and three downstream. The control mesocosm was
110 homogeneous with sand and gravel as the sediment, no canopy cover, uniform depth of over 30
111 cm in pools, negligible flow and ambient temperature. The heterogeneous mesocosm was
112 modified to have different habitats up and downstream to mimic a natural stream gradient.
113 Within the headwater portion, the sediment was composed of cobble and gravel in the riffles and
114 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.

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



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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.

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Ten fish of both sex and species, 40 total, were randomly distributed among each pool

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within each mesocosm. This gave way to a grand total of 120 fish throughout all three

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mesocosms. A 24-hour period was given to allow the fish to acclimate to the new environments.

132

After acclimatization, one GoPro camera was placed over each tank. To avoid bias, the choice of

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which treatment to use during each observed time was chosen at random. The placement of the

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cameras during each observation, morning or afternoon, on either the control, heterogeneous or

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homogeneous treatments was also random to avoid systematic bias. These cameras took one

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picture every five minute for two hours. This happened twice daily between 9am-11am and again

137 from 2pm-4pm for eight days completing one trial. A total of three trials (each with eight days of
138 observation) were completed.

139 *Data Analysis*

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

154

155 **Results**

156 Cameras recorded pictures for a total of 48 two-hour periods (15 heterogeneous, 16
157 control, and 17 temperature only). The unbalanced design was a result of some trials being
158 discarded because weather conditions did not yield pictures for which I could reliably identify
159 individual fish. This was usually due to rain disrupting the water surface or overcast conditions

160 producing glare that blocked portions of the pools. There was a total of 34,560 photos. Due to
161 time constraints, I analyzed every fifth photo (five minute intervals) to detect the presence of the
162 tagged fish in each location. There was a total of 7,200 photos analyzed, 2,400 per trial, yielding
163 a total of 3,016 individual fish observations (Fig. 3). The number of fish observed was consistent
164 across treatments (control: 783, heterogeneous: 1027, and temperature only: 1206) and averaged
165 62.8 observations for each two-hour observation period. One unexpected trend was the disparity
166 in the number of observations for males vs. females. Despite the sex ratios being equal in all
167 treatments, there were 2032 observations of females and 984 observations of males. This is most
168 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*.

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

175 There were a total of 1513 *F. notatus* (975 females and 538 males) observed in the
176 photos. With this data, six t-test were run to test for differences between both sex and treatment.
177 The means and standard error gathered from these t-test were then formatted into a bar graph
178 with error bars (Fig. 4). When comparing the means and standard error of each species by sex to
179 that of the control there was variability as expected. Overall, *F. notatus* responded strongly to the
180 experimental gradient with index scores in the control closer to 0 (averaged $\bar{x} = 0.1981 \pm$
181 0.0716) compared to the heterogeneous where most individuals were found in downstream
182 habitat (averaged $\bar{x} = 0.5354 \pm 0.0595$). Male *F. notatus* seemed to have a stronger response than
183 females, but these results were not significantly different (heterogeneous averaged $\bar{x} = 0.6333 \pm$
184 0.0639 , temperature only averaged $\bar{x} = -0.2021 \pm 0.1186$, control averaged $\bar{x} = 0.2263 \pm 0.0944$).

185 There were a total of 1503 *F. olivaceus* (1057 females and 446) observed. *F. olivaceus*
186 did not respond as strongly to the gradient as *F. notatus*. Female index values increased
187 marginally from the control (averaged $\bar{x} = 0.1914 \pm 0.0826$) to heterogeneous (averaged $\bar{x} =$
188 0.3247 ± 0.0970). Male *F. olivaceus* decreased in heterogeneous treatment (averaged $\bar{x} = 0.2475$
189 ± 0.0918) when compared to the control (averaged $\bar{x} = 0.3052 \pm 0.1113$) (Fig. 5).

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192 **Table 1.** Results from a repeated measures ANOVA testing for differences in spatial distribution
193 between species, treatment, and sex. Statistical significance at $P < 0.05$ is indicated with an
194 asterix.
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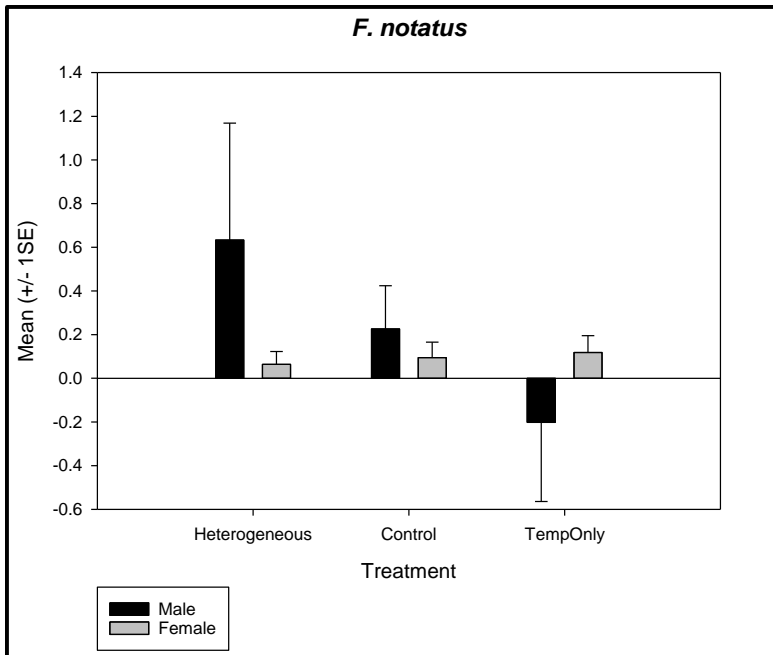
Interaction	Sum Sq.	Mean Sq.	<i>df</i>	<i>F</i>	<i>P</i>
Species	0.584	0.5844	1	1.189	0.27621
Treatment	52.732	26.3662	2	53.646	2.00E-16*
Sex	0.79	0.7903	1	1.608	0.20556
Species x Treatment	4.566	2.283	2	4.645	0.01017*
Species x Sex	0.283	0.2826	1	0.575	0.44874
Treatment x Sex	0.142	0.071	2	0.144	0.86558
Species x Treatment x Sex	0.919	0.4594	2	0.935	0.39361

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

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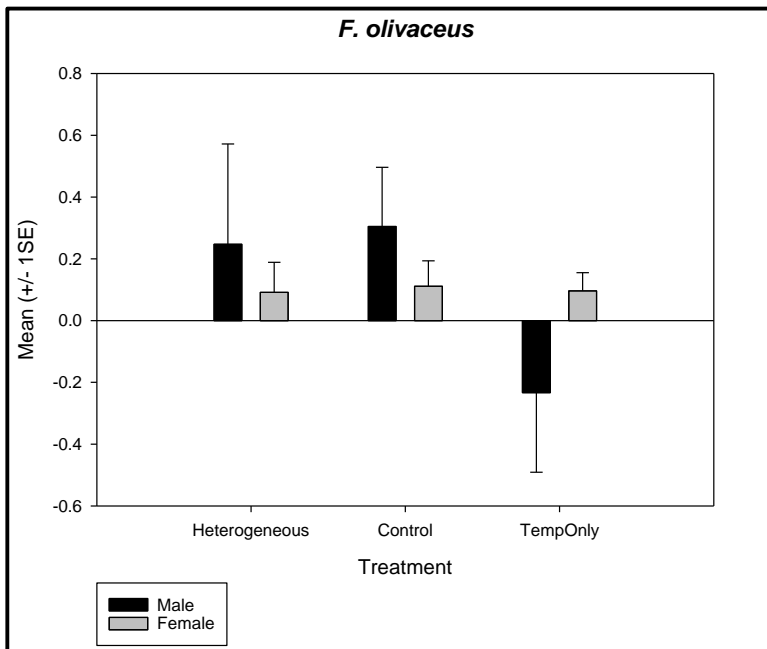
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Figure 4. Mean and standard error index values for male and female *F. notatus* in the heterogeneous, control and temperature-only treatments. Index values of 0 indicate even distribution upstream and downstream. Negative and positive values indicate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



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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.

213 **Discussion**

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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).

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

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

257 The method of obtaining photos may have led to a sampling bias. Having the GoPro
258 attached to a wooden arm allowed for full view of each tank. However, some photos had shadow
259 areas around the edges of the tank limiting the field of view and possibly allowing for incorrect

260 species count. Sun glare and shadows from artificial canopy cover also may have played a small
261 role in possible sampling bias.

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

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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 EXPIRATION DATE: September 30, 2018

Frank Moore, PhD
IACUC Chair

10/01/2015

Date

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297

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