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Nathaniel Steffensmeier

Naznin Sultana Remex

Robert Hrabik

David D. Duvernell
Missouri University of Science and Technology, duvernelld@mst.edu

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Status of the Blackstripe (Fundulus notatus) and Blackspotted (F. olivaceus) Topminnows in the Ozark uplands of Central Missouri

Nathaniel Steffensmeier
The Ohio State University, steffensmeier.8@buckeyemail.osu.edu

Naznin Sultana Remex
Missouri University of Science and Technology, nrqd7@mst.edu

Robert Hrabik
Missouri Department of Conservation, robert.hrabik@mdc.mo.gov

David D. Duvernell
Missouri University of Science and Technology, duvernelld@mst.edu

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Abstract

The topminnow species *Fundulus notatus* and *F. olivaceus* have broadly overlapping geographic distributions that extend throughout much of the central and southern United States. In the northern portion of their respective ranges, in Missouri, the regional distributions of the two species coincide largely with recognized ecoregions. In the unglaciated southern half of Missouri, *F. olivaceus* is distributed throughout Ozark upland habitats while *F. notatus* is abundant in marginal large river and prairie habitats along the Ozark borders. An exception to this partitioning is the historical report of abundant *F. notatus* in the Bourbeuse and upper Meramec River drainages within the Ozark uplands ecoregion. We conducted an extensive survey of the Bourbeuse and Dry Fork Meramec Rivers to determine topminnow species composition in these systems. Our surveys found abundant *F. olivaceus* populations throughout these drainages and failed to uncover any *F. notatus* individuals. A review of museum accessions from the 1940s and 1960s confirms the historical presence of *F. notatus* in these river drainages, suggesting that a significant shift in topminnow species abundance has occurred in the past half century.

Keywords

ecoregion, species abundance, hybridization, species replacement

Cover Page Footnote

We are grateful to collections managers who shared images of specimens from collections at the University of Kansas (Andy Bentley), University of Michigan (Douglas Nelson), Oregon State University (Brian Sidlauskas), Ohio State University (Mark Kibbey), Cornell University (Casey Dillman), and the Illinois Natural History Survey (Chris Taylor). We wish to thank Jacob Schaefer for discussions of this work. NS was supported by an internship provided by the National Great Rivers Research and Education Center. Funding for this project was provided by NSF grant DEB-1556778.

INTRODUCTION

Topminnows of the *Fundulus notatus* species complex are among the most widely distributed members of the Cyprinodontiform genus *Fundulus*. This group is of particular interest to ichthyologists and phylogeographers because member species have broadly overlapping geographic distributions and occupy similar ecological niches, yet co-occurrence within habitats is generally limited to ecological transition zones suggesting competitive exclusion (Duvernell and Schaefer, 2014; Thomerson and Wooldridge, 1970). The Blackstripe Topminnow (*F. notatus*) is distributed throughout the Gulf Coastal Plain from central Texas to Alabama, as far north as formerly glaciated regions of southern Michigan and Wisconsin and northern Iowa, and as far west as central Kansas (Thomerson, 1966). The Blackspotted Topminnow (*F. olivaceus*) exhibits a broadly overlapping distribution that extends from east Texas to Florida, but only as far north as the Shawnee Hills of southern Illinois, and the Ozark Highlands of southern Missouri (Thomerson, 1966). The unusually broad overlapping distribution of these two closely related species led Wiley and Mayden (1985) to cite them as an illustrative example of sister species whose overlapping ranges indicated recent range expansions. Phylogeographic analysis of nuclear and mitochondrial variation support a post-Pleistocene northward range expansion for both species (Duvernell et al., 2013; Duvernell et al., 2019).

Both species occupy a wide range of stream habitats throughout their respective ranges (Thomerson, 1966). In the northern portions of their ranges, *F. notatus* is most commonly found in low gradient tributaries and margins of large rivers, and prairie stream habitats, which typically exhibit finer silt substrates. In contrast, northern latitude *F. olivaceus* tend to be restricted to high gradient upland stream habitats with gravel substrates (Braasch and Smith, 1965). Generally, the Ozark upland streams in the southern half of Missouri are inhabited primarily by *F. olivaceus*, while *F. notatus* is largely excluded from upland Ozark habitats, occurring in formerly glaciated regions of east-central Missouri north of the Missouri River, marginal habitats along the Mississippi River, the coastal plain Bootheel region of southeast Missouri, and prairie streams in the headwaters of the Osage and Spring Rivers in southwest Missouri (Pflieger, 1997).

A notable exception to the exclusion of *F. notatus* from Ozark upland habitats is the reported presence of *F. notatus*, and its co-occurrence with *F. olivaceus*, in headwater streams of the Bourbeuse and Meramec River drainages.

After initial unsuccessful attempts to sample *F. notatus* in this drainage system, we undertook a comprehensive survey of tributaries of the Bourbeuse River and upper Meramec River to update the status, and ascertain the current distribution of *F. notatus* in this drainage system. We have employed nuclear and mitochondrial diagnostic genetic markers to unequivocally identify sampled individuals to species. We also reviewed voucher specimens in research collections of *F. notatus* and *F. olivaceus* dating back to 1930 to confirm the historical presence of *F. notatus* in this drainage system.

MATERIALS AND METHODS

We collected *Fundulus* sp. using dipnets from all headwater stream and main stem river habitats in the Bourbeuse River, as well as the Dry Fork of the Meramec River, where museum database records indicate the presence of *F. notatus*. For each specimen, a small fin clip was preserved in 100% ethanol for genetic analysis before the fish was preserved in 10% formalin in individual tubes with matching labels. Additional sampling was conducted throughout central and southern Missouri to obtain reference collections of each species (Fig. 1; Table 1).

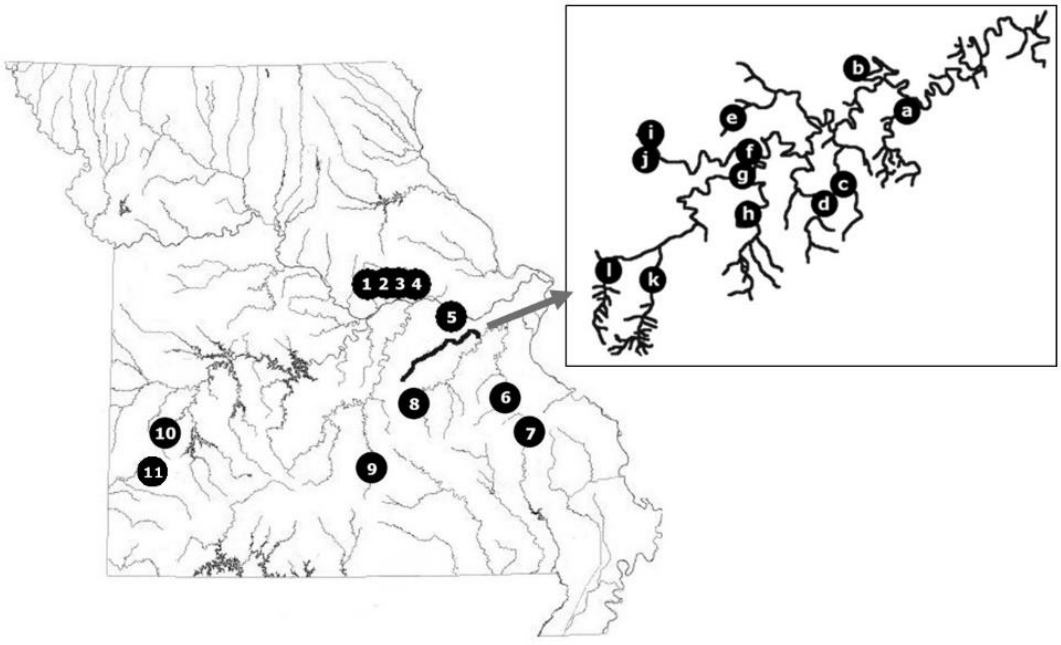

Figure 1. Map of localities for specimens that were collected for morphological and genetic analysis in this study. The collection sites correspond to collection information provided in Table 1.

Species-diagnostic molecular nuclear and mitochondrial DNA markers were employed to determine species and possible hybrid status for each individual. DNA was extracted using Qiagen blood and tissue extraction columns (Qiagen Inc.). Nuclear and mitochondrial DNA markers were assayed using previously validated *F. notatus* species-diagnostic single nucleotide polymorphisms (SNPs). SNPs at two nuclear intron and one mitochondrial loci were assayed using a polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) assay (Table 2). Thermal cycling conditions included an initial denaturing step at 95 C for 5 min, thirty cycles of 94 C for 30 s, 58 C for 30 s, 72 C for 1 min, and a final extension of 72 C for 5 min. Amplified DNA fragments were digested with an appropriate restriction enzyme (Table 2) and the digested products were electrophoresed on a 2% agarose gel in 1X TAE buffer.

Previous studies have demonstrated the lack of reliable meristic characters (Braasch and Smith, 1965; Thomerson, 1966) and morphometric measurements (Schaefer et al., 2011a) for species identification. Presence or absence of sharply defined spots in males is the most reliable morphological characteristic for differentiating between *F. olivaceus* and *F. notatus* (Fig. 2). However, diagnostic field markings can be challenging to interpret, even for experienced ichthyologists. In *F. olivaceus,* the presence of clearly defined spots is a sexually dimorphic characteristic, occurring most prominently along the full length of the dorsal body surface in sexually mature males (Schaefer et al., 2012). Spot density is typically lower in females. In *F. notatus,* spots are either absent or present to a limited extent in the caudal region. When present in *F. notatus,* spots are relatively diffuse in appearance in both sexes (Schaefer et al., 2012). The presence of spots on the dorsal, anal and caudal fins is not diagnostic, as males of both species often exhibit clearly defined spots on the fins.

Sexually mature specimens collected and genotyped in this study were photographed for morphological analysis. Spots were counted on the left dorsal body surface, above the lateral line, of sexually mature males and females, and spot density was standardized by standard length (SL) for each individual. We reviewed photographs of specimens held in the Kansas University and University of Michigan ichthyology collections to assess spot phenotypes. The quality of preservation was variable, and many images were not of sufficient clarity for quantitative assessment of spot counts. We resorted to a qualitative assessment of spot presence/absence to evaluate species assignment in those cases. All specimens in research collections were fixed in formalin rendering them unusable for genetic analysis.

Table 1. Sampling locations for *F. notatus* and *F. olivaceus* collections. Sample sizes are reported as the number of genotyped individuals exhibiting indicated ratios of *F. olivaceus*/*F. notatus* diagnostic nuclear alleles.

Stream	Latitude	Longitude	*oli* 4/0	3/1	2/2	1/3	*not* 0/4
Bourbeuse							
a. Spring Cr.	38.3236	-91.1574	4	0	0	0	0
b. Big Cr.	38.3893	-91.2475	5	0	0	0	0
c. Boone Cr.	38.2150	-91.2690	4	0	0	0	0
d. L. Bourbeuse R.	38.1966	-91.3115	4	0	0	0	0
e. Red Oak Cr.	38.3120	-91.4783	5	0	0	0	0
f. Dry Fork	38.2652	-91.4435	4	0	0	0	0
g. Bourbeuse R.	38.2348	-91.4565	5	0	0	0	0
h. Brush Cr.	38.1772	-91.4477	7	0	0	0	0
i. Dry Fork	38.2876	-91.6367	5	0	0	0	0
j. Dry Fork	38.2628	-91.6428	6	0	0	0	0
k. Bourbeuse R.	38.1182	-91.7006	5	0	0	0	0
l. Robinson Cr.	38.0392	-91.5890	5	0	0	0	0
1. Middle R.	38.8443	-92.0284	0	0	0	1	4
2. Auxvasse Cr.	38.7959	-91.8766	0	0	0	1	9
3. L. Tavern Cr.	38.7177	-91.6599	0	0	0	1	2
4. Loutre R.	38.8108	-91.5024	5	0	0	0	0
5. Boeuf Cr.	38.5074	-91.3115	6	0	0	0	0
6. Big R.	37.7553	-90.8846	15	0	0	0	0
7. St. Francis R.	37.5553	-90.3571	25	0	0	0	6
8. Dry F. Mer. R.	37.8527	-91.6645	13	0	0	0	0
9. Big Piney R.	37.3331	-91.9582	16	0	0	0	0
10. Horse Cr.	37.7224	-93.9964	7	0	2	3(2)*	3(2)*
11. N. F. Spring R.	37.4295	-94.0936	0	0	1	0	3

*Numbers in parentheses indicate individuals with a majority of *F. notatus* nuclear alleles, but *F. olivaceus* mtDNA.

Table 2. Oligonucleotide sequences and restriction enzymes used to genotype *Fundulus* sp.

Locus	Oligonucleotide (5'-3')			R. E.	Cuts Reference
CIRP	For GCTTCGAGACCAACGAAGAC Rev CGTCACGATACGATCCAGAG	*Alu*I	*F. notatus*[a]	(Schaefer et al., 2011b)	
GPX	For AGGTGAGGAAACCCACCTTT Rev TAGCGGCCTCTCTCATGTTT	*Hae*III	*F. olivaceus*	(Schaefer et al., 2011b)	
Cyt *b*	For TCGCACATATTTGCCGTGAT Rev CCCTCAGAAGGAYATTTGACCTCA	*Stu*I	*F. notatus*	(Song et al., 1998)	

[a] Restriction enzyme cuts multiple invariant sites shared between both species in addition to the species-diagnostic site.

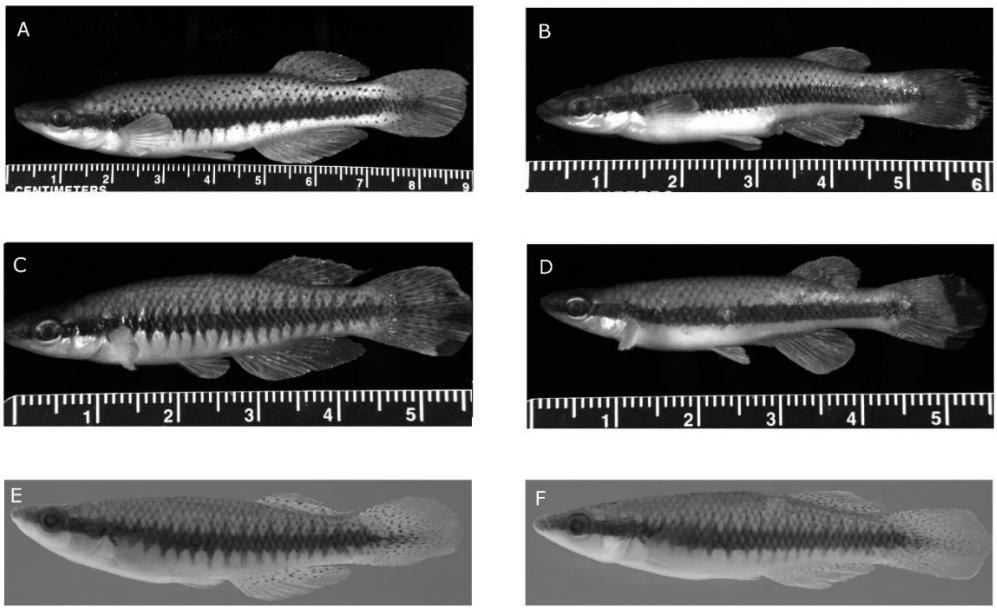

Figure 2. Images of select male and female individuals collected and reviewed in this study. A) Dry Fork of Bourbeuse River male *F. olivaceus*; B) Little Bourbeuse River female *F. olivaceus*; C) Auxvasse Creek male *F. notatus*; D) Auxvasse Creek female *F. notatus*; E) Little Bourbeuse River male *F. notatus* (1963; KU 10617); F) Pleasant Valley Creek male *F. notatus* (1963; KU 10620).

RESULTS

We genotyped 59 individuals from 12 sites within the Bourbeuse River drainage, and an additional 121 individuals from 11 drainages throughout Missouri. Sampling efforts in the Bourbeuse River included all major tributary streams, as well as Dry Fork Meramec River, where *F. notatus* individuals were previously reported (Pflieger, 1975). Reference streams included five small direct tributaries of the Missouri River, four additional river drainages in the southern Missouri Ozarks, and two prairie streams in western Missouri. All individuals sampled in the Bourbeuse River exhibited the *F. olivaceus* genotype at both nuclear and mitochondrial loci, as did all individuals sampled from the Meramec River, the Big River, and the Big Piney River. The St. Francis River sample included both *F. olivaceus* and *F. notatus* genotypes from a single site, but no putative hybrids. Among Missouri River tributaries, the Loutre River and Bouef Creek both exhibited *F. olivaceus* genotypes, while the Middle River, Auxvasse Creek and Little Tavern Creek contained *F. notatus* genotypes. One individual each from the Middle, Auxvasse, and Little Tavern drainages were heterozygous at the CIRP gene locus. This likely represents a low frequency polymorphism in the *F. notatus* gene pool, as was previously reported in a small number of populations in the northern

distribution of *F. notatus* (Duvernell and Schaefer, 2014). Presence of low frequency heterozygous genotypes at the CIRP locus in these *F. notatus* populations is not indicative of recent hybridization. Samples from Horse Creek, a headwater tributary of the Osage River, included both species and a number of putative hybrids, while samples from the geographically proximate North Fork Spring River exhibited three individuals with *F. notatus* genotypes, and one putative hybrid. Putative hybrid ancestry was indicated by frequent heterozygosity at one or both nuclear loci, a mismatch in species-specific alleles between the two nuclear loci, or mismatch between the nuclear and mitochondrial loci.

We quantified spot density in mature males and females in Bourbeuse River and other drainage reference sites. A box plot of spot densities from each location illustrated high and low spot densities among samples, matching genetic data (Fig. 3). Genetically assigned *F. olivaceus* and *F. notatus* individuals were plotted separately for St. Francis River and Horse Creek samples. The difference between spot densities measured in males versus females reflected sexual dimorphism in *F. olivaceus*. The distribution of spot densities in Bourbeuse River specimens from 1963 overlapped with those of *F. notatus* populations, and not contemporary Bourbeuse River specimens (Fig. 3).

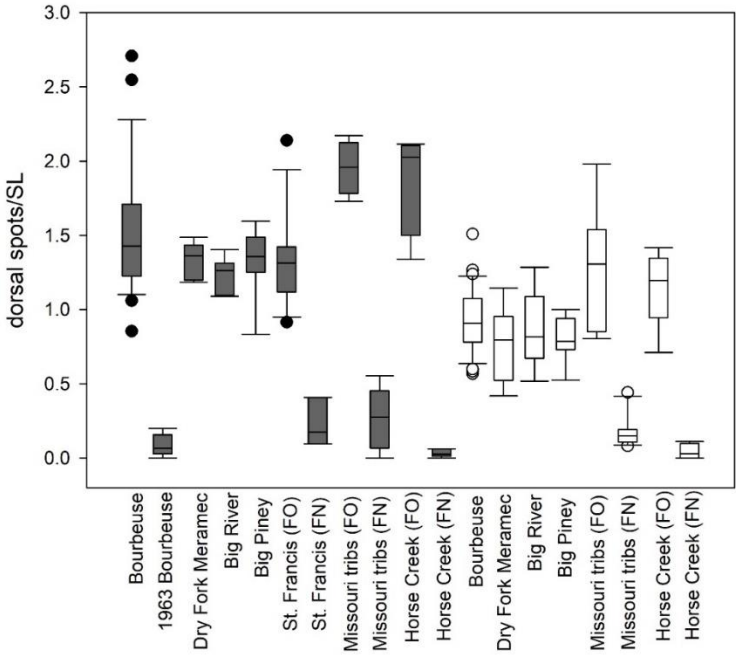

Figure 3. Box plot of spot density (spots/mm SL) for *F. olivaceus* (FO) and *F. notatus* (FN) collections from this study, and from specimens collected in 1963. Males are reported with shaded symbols and females are reported with white symbols.

We reviewed specimens collected in 1941 and 1963 from throughout the Bourbeuse River drainage, and the western-most tributary of the Meramec River drainage. All reviewed specimens had previously been identified as *F. notatus*. We confirmed individuals whose spot phenotypes were distinctively different from Bourbeuse River specimens collected in this study for six collections, including the Bourbeuse River and three of its tributaries (Table 3). For the remaining collections, specimens were either too degraded to make an assessment of the spot phenotype, or individuals exhibited spot phenotypes that were determined to be consistent with *F. olivaceus*. We also reviewed some lots of specimens from the Bourbeuse River drainage collected more recently than 1963, but found no convincing examples of *F. notatus*.

Table 3. Research collections reviewed in this study, and morphological assessment of species identification in each collection.

Accession	Stream	Drainage	County	N	Year	Species
KU 10648	Dry Fork	Bourbeuse	Maries	3	1963	*F. notatus*
KU 10620	Pleas. Val. Cr	Bourbeuse	Crawford	3	1963	*F. notatus*
KU 10617	Little Bourbeuse R.	Bourbeuse	Crawford	3	1963	*F. notatus*
UMMZ 148344	Dry Fork	Bourbeuse	Gasconade	6	1941	*F. notatus*
UMMZ 149567	Red Oak Creek	Bourbeuse	Gasconade	7	1941	uncertain
UMMZ 148376	Bourbeuse River	Bourbeuse	Gasconade	7	1941	*F. notatus*
UMMZ 148393	Brush Creek	Bourbeuse	Crawford	2	1941	*F. notatus*
UMMZ 149594	Clear Creek	Bourbeuse	Phelps	4	1941	uncertain
UMMZ 149613	Bourbeuse River	Bourbeuse	Phelps	2	1941	uncertain
UMMZ 149653	Bourbeuse River	Bourbeuse	Franklin	4	1941	*F. olivaceus*†
UMMZ 149704	Birch Creek	Bourbeuse	Franklin	3	1941	uncertain
UMMZ 149567	Dry Fork	Meramec	Phelps	5	1941	*F. olivaceus*†
UMMZ 149510	Dry Fork	Meramec	Dent	5	1941	*F. olivaceus*†

†At least some individuals in the lot exhibited a spot phenotype consistent with *F. olivaceus* while others may have been equivocal. No individuals exhibited an unequivocal *F. notatus* phenotype.

DISCUSSION

The distribution and relative abundance of *F. notatus* and *F. olivaceus* in the Missouri Ozarks presents an interesting challenge to our understanding of the ecological and environmental factors that determine the distributions of these topminnow species in the northern portions of their respective ranges. Throughout most of Missouri, *F. olivaceus* is abundant in typical Ozark upland habitats, with *F. notatus* occurring primarily in the large river and plains ecoregions. A significant exception is the reported historical abundance of *F. notatus* throughout the Bourbeuse River, and the western most headwaters of the Meramec River, both rivers comprising a drainage that lies entirely within the upland Ozarks ecoregion.

In the first printing of the definitive *The Fishes of Missouri* (Pflieger, 1975), distributional maps based on research collections indicated that the Bourbeuse and upper Meramec rivers were exclusively inhabited by *F. notatus*. In the second printing of *The Fishes of Missouri*, Pflieger (1997) amended the distributional maps to indicate the presence of both *F. notatus* and *F. olivaceus* in these rivers. Our sampling and genotyping efforts throughout the Bourbeuse River and the Dry Fork in the headwaters of the Meramec River failed to uncover any evidence of *F. notatus*. Nuclear and mitochondrial genotypes definitively assigned all individuals to *F. olivaceus*, and all adult males exhibited spotted phenotypes indicative of *F. olivaceus.*

Our own sampling efforts contrasted with collections from 1941 and 1963. Upon review of these collections, we found clear evidence of *F. notatus* specimens in at least six collections from four different tributaries as well as the mainstem of the Bourbeuse River. Notably, we did not find any convincing evidence of *F. notatus* in collections made after 1963, regardless of how they were accessioned. The evidence is convincing that there has been a drainage-wide shift in the relative abundance of topminnow species in the Bourbeuse and Meramec rivers, though a mechanism is not apparent. We were unable to document any contemporaneous *F. notatus* in the entire Bourbeuse River drainage. Future ichthyo-faunal surveys of the Bourbeuse River and the headwaters of the Meramec River will be important for determining if *F. notatus* is present, and may be useful for delineating the determinants of topminnow species abundance in drainages where both species co-occur.

The Bourbeuse and Meramec River drainages are not the only places where topminnow species distributions may be shifting in Missouri. In our collection from the Little St. Francis River, a tributary in the upper portion of the St. Francis River, we documented the presence of *F. notatus* at a local relative abundance of about 20% in a mixed sample of both topminnow species. While *F. notatus* has been extensively documented in the lower St. Francis River drainage, there are no published reports of *F. notatus* in the upper reaches of the drainage, or in the tributary where we sampled. The lower St. Francis River drainage, below Lake Wappapello, lies within the big river ecoregion habitat of the Gulf Coastal Plain, while the headwater of the drainage, including the Little St. Francis River, lies within the Ozark upland region.

Finally, the contrast in the extent of hybridization observed in this study, where hybrids were observed to be relatively abundant in Horse Creek, but apparently absent in the Little St. Francis River, mirrors species distribution-wide variation in the abundance of hybrids in contact zones (Duvernell and Schaefer,

2014). The underlying basis for reproductive isolation between these species includes both pre- and post-zygotic mechanisms (Vigueira et al., 2008), and reasons for variation in extent of hybridization among contact zones have been attributed to ecological factors and transition zone structure (Duvernell and Schaefer, 2014).

DISPOSITION OF SPECIMENS

Specimens and materials collected for this study have been vouchered in the Ichthyology Collection at the Biodiversity Institute at University of Kansas (Specimens: KUI 41892-KUI 41927; Tissues: KUIT 11039-KUIT 11111). All specimens were collected under the authority of a research collecting permit issued by the Missouri Department of Conservation.

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