Summer 7-28-2022

Chemical and Biological Stressors Threaten Native Fish Diversity in the Spring River Subbasin of Kansas

Alexandra King
Pittsburg State University, aking@gus.pittstate.edu

CHEMICAL AND BIOLOGICAL STRESSORS THREATEN NATIVE FISH DIVERSITY IN THE SPRING RIVER SUBBASIN OF KANSAS

A Thesis Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Degree of Master of Science

Alexandra D. King

Pittsburg State University

Pittsburg, KS

August, 2022

CHEMICAL AND BIOLOGICAL STRESSORS THREATEN NATIVE FISH DIVERSITY IN THE SPRING RIVER SUBBASIN OF KANSAS

Alexandra King

APPROVED:

Thesis Advisor ______________________________________

James E. Whitney, Department of Biology

Committee Member ______________________________________

Anuradha Ghosh, Department of Biology

Committee Member ______________________________________

Philip Rudd, Department of English and Modern Languages

ACKNOWLEDGEMENTS

I want to begin by thanking my thesis advisor, Dr. James Whitney, for always going above and beyond what was necessary, whether it was helping me find funding sources for my research or providing guidance and advice throughout my graduate studies. I am extremely grateful for my committee members Dr. Anuradha Ghosh and Dr. Philip Rudd, and I want to give many special thanks to Dr. Ghosh for teaching me how to do DNA extractions and PCR-RFLP genetic analyses in the laboratory and for all the time and effort she put into guiding me through graduate teaching. I am grateful for Dr. Chung for giving me v*alu*able advice and Master mix, and many thanks goes to Kim Grissom for teaching me how to properly use prep lab equipment. I would like to thank not only Dr. Ghosh, but also Dr. Rider for letting me use your equipment and giving me permission to work in your labs. I want to give special thanks to my predecessor, Kali Boroughs, for providing me with contemporary sampling data, and all the fisheries technicians, Joshua Holloway, Aliyah Clemens, and Austin Thompson, who helped sample riffle fishes from 2019-2021. I want to thank Blake Hansen, Caitlyn Aymami, and Olivia Rode for volunteering and assisting with riffle fish sampling efforts in the Spring River subbasin during the summer of 2021. This research would not have been possible without funding from the U.S. Fish and Wildlife Service (Grant #F19AF00039) and the Pittsburg State University Department of Academic Affairs, and the assistance of Jordan Hofmeier from the Kansas Department of Wildlife and Parks. I send my gratitude and appreciation out to all the generous private landowners who provided us with permission to sample on their property, and lastly, I want to thank my family, friends, and fur babies for providing me with emotional support while completing this journey.

Fish sampling was conducted under the U.S. Fish and Wildlife Service Recovery Permit #TE13623D-0 and State of Kansas Scientific Collection Permits #SC-014-2019, #SC-041-2020, and SC-039-2021.

CHEMICAL AND BIOLOGICAL STRESSORS THREATEN NATIVE FISH DIVERSITY IN THE SPRING RIVER SUBBASIN OF KANSAS

An Abstract of Thesis by
Alexandra King

Chemical pollution and nonnative species are two of the major threats facing freshwater fishes. As such, understanding how freshwater fish communities respond to pollution remediation efforts and nonnative fish introductions are primary goals of native fish conservation. Within the Spring River subbasin (SRS) of southeastern Kansas, this study examined the long-term response of riffle fish communities to decreases in heavy metal concentrations, as well as the effects of introduced Blackspotted Topminnow (*Fundulus olivaceus*) on native Blackstripe Topminnow (*Fundulus notatus*). The objectives of our research in Chapter 01 of this thesis were to quantify changes in riffle fish community structure across a temporal pollution severity gradient that spanned 1993-1995 (more severe) to 2019-2021 (less severe), and in Chapter 02 we used genetic analyses to identify *Fundulus* spp. distributions as well as their hybridization frequency. Since the 1990's, declining heavy metal concentrations from mining remediation have had a positive response on pollution sensitive riffle fish species, including the federally threatened Neosho Madtom (*Noturus placidus*). In response to this long-term water quality improvement, riffle fish community structure shifted from predominately pollution tolerant species to pollution intolerant species, many of which are species of greatest conservation need (SGCN) in Kansas. This research suggested that pollution legislation designed to improve water quality creates a ripple effect that has the power to

stimulate the recovery of imperiled fish species. Regarding *Fundulus* spp. distributions, we found that after at least 20 years of being introduced to the SRS, the Blackspotted Topminnow remained largely restricted the stream where it was initially found in 2000 (i.e., Shoal Creek) and had not spread to the rest of the SRS other than the Spring River near its confluence with Shoal Creek, which coincides with a small cooling reservoir for a coal-fired powerplant near Riverton, KS (i.e., Empire Lake). Therefore, Empire Lake may have acted as a dispersal barrier against further Blackspotted Topminnow invasion, allowing native Blackstripe Topminnow to remain the dominant *Fundulus* spp. throughout the rest of the SRS. However, the limited spread of Blackspotted Topminnow may also be explained by variation in water clarity among streams in the SRS resulting from differences in land use and cover, as widespread agriculture may cause much of the SRS to be too turbid to support Blackspotted Topminnow. In contrast, Shoal Creek is more forested. Furthermore, the hybridization frequency between *Fundulus* species was greatest in the Spring River directly below and Shoal Creek directly above Empire Lake. Though nonnative Blackspotted Topminnow has hybridized and displaced native Blackstripe Topminnow in Shoal Creek and the lower Spring River directly below Empire Lake, it appears at present that this invasion is contained to Shoal Creek and the Spring River directly below Empire Lake. However, other Ozarkian tributaries of the Spring River where Blackspotted Topminnow have yet to invade may have the clear water that is required for their successful establishment, thus monitoring and public outreach is necessary to help prevent the invasion of these streams. Our results demonstrated a conservation success story for native fishes of the Spring River regarding the increased prevalence of pollution-sensitive and oftentimes imperiled species following water quality improvements. Yet, more conservation actions may be necessary in the SRS to help control nonnative Blackspotted Topminnow and restore displaced Blackstripe Topminnow to Shoal Creek.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I

CLEANSING OUR WATERS: HOW RIFFLE FISH COMMUNITIES IN THE SPRING RIVER OF KANSAS RESPONDED TO POLLUTION LEGISLATION AND REMEDIATION

Introduction

Pollution is harmful to stream organisms, as a multitude of pollutants enter streams via numerous pathways. However, input pathways can be placed into two major categories in stream ecosystems, including point source and non-point source (Whitney et al. 2019). Point source pollution is derived from a known origin (e.g., municipal sewage effluent, industrial waste discharge, and mine drainage), whereas with non-point source pollution the input location remains unknown (e.g., agricultural and urban runoff; atmospheric deposition) (Katz and Gaufin 1953; Pucket 1995). The highest pollution concentrations usually occur at the initial input site and the effects of pollution tend to attenuate downstream in lotic ecosystems since tributaries and groundwater help dilute pollution (Hughes and Gammon 1987; Ryon 2011). In the United States two major pieces of legislation were enacted to clean up waterbodies, including the Clean Water Act (CWA) amended in 1972 and the Surface Mining Control and Reclamation Act

(SMCRA) of 1977. These pieces of legislation have reduced point source pollution but have been less effective at controlling non-point source pollution. Although these legislative efforts aimed at reducing water pollution have been in place for >40 years, few studies have examined how aquatic organisms have responded to resultant water quality improvements. As such, a lingering question remains: how have fishes responded to these pollution regulation efforts?

Not all fishes are created equal, as some fish species are more sensitive to water pollution than others (Fausch et al. 1990; Karr et al. 1986; Whitney et al. 2019). For instance, there are tolerant species which can withstand higher concentrations of pollutants, compared to intolerant species that are more sensitive. Greater physiological tolerance and generalist ecological strategies (e.g., omnivore-detritivores; r life history strategy) are present in more pollution tolerant fishes; in contrast, intolerant fishes have a more sensitive physiology and specialized ecological requirements (Fausch et al. 1990; Grabarkiewicz and Davis 2008; Whitney et al. 2019). Furthermore, habitat preference is often related to pollution tolerance, as more stagnant pools often have poorer water quality (e.g., higher temperatures; lower dissolved oxygen; pollutant deposition) compared to faster-flowing riffles, resulting in pool fishes tending to be more pollution-tolerant compared to riffle species (Brabec et al. 2004). As such, focusing on responses of riffle fishes to pollution remediation can yield more definitive insight regarding biotic responses to water quality improvements, given the greater pollution-sensitivity of riffle fishes. Lastly, these tolerance categories often have conservation implications, as many imperiled species are pollution intolerant (Grabarkiewicz and Davis 2008; Jelks et al. 2008; Whitney et al. 2019), allowing them to serve as essential biological indicators.

High concentrations of heavy metals such as cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn) can reduce fish population densities and ultimately fish diversity (Allert et al. 2013). Fishes absorb heavy metals directly through their gills and integumentary system, and indirectly via bioaccumulation from consuming lower trophic levels contaminated by metals (i.e., invertebrates, primary producers) (Authman et al. 2015; Zeitoun and Mehana 2014; Boroughs 2020). Heavy metals not only reduce fish health and reproductive fitness, but they can also alter the structure and quality of habitats and food sources (Vuori 1995). These deleterious impacts of toxic metals can extend beyond aquatic environments into terrestrial ecosystems when terrestrial organisms, including humans, consume aquatic organisms tainted by heavy metals (Park et al. 2020).

The Spring River drains parts of southwest Missouri, southeast Kansas, and northeast Oklahoma, which includes a large portion of the Tri-State Mining District. From 1850 to 1950 the Tri State Mining District was one of the primary sources of lead and zinc in the world (Juracek 2006). A legacy of this mining is that the Spring River and several of its eastern Ozarkian tributaries (i.e., Center Creek, Turkey Creek, Short Creek, and Shoal Creek; Fig. 1) receive heavy metal inputs (i.e., Cd, Cu, Pb, and Zn) from abandoned mines. Historically, this resulted in the Spring River mainstem below Center Creek having greater toxic metal concentrations compared to the Spring River above Center Creek (Wildhaber et al. 2000; Chambers et al. 2005; Angelo et al. 2007; Boroughs 2020). However, in response to pollution remediation resulting from legislation previously described (i.e., CWA and SMCRA), water quality in the lower Spring River and its Ozarkian tributaries has greatly improved over time (Fig. 2; Boroughs 2020). Although, in certain Spring River segments pollutants remain elevated above reference conditions set by the Environmental Protection Agency (Fig. 2; Boroughs 2020). Thus, it remains unclear if pollution abatement has been substantial enough to elicit a response in pollution-sensitive fishes.

The Spring River is home to many imperiled riffle fish species, including the federally threatened Neosho Madtom (*Noturus placidus*), which was listed under the Endangered Species Act in 1990 (USFWS 1991). Riffle-dwelling fishes in the genera *Noturus*, *Etheostoma,* and *Cottus* are particularly sensitive to heavy metal pollution via direct and indirect effects and are often the first to disappear following environmental degradation (Boschung and Mayden 2004; Grabarkiewicz and Davis 2008; Allert et al. 2009; Boroughs 2020). For instance, Allert et al. (2009) found that Banded Sculpin (*Cottus carolinae*) densities were significantly reduced at sites impacted by mining compared to sites positioned upstream of mining inputs. Furthermore, in King et al. (2021a), Banded Sculpin and Sunburst Darter (*Etheostoma mihileze*) were mostly restricted to small tributaries in the Spring River subbasin that were less impacted by heavy metal pollution. Therefore, further monitoring is necessary to investigate whether pollution legislation and mining remediation have successfully cleansed our waters.

The purpose of this study is to compare riffle fish communities in the Spring River subbasin across two time periods positioned along a temporal gradient of pollution severity, including 1993-1995 (more severe pollution) and 2019-2021 (less severe pollution). We will examine how the overall riffle fish community has changed in response to improving water quality via mining remediation, in addition to responses of various components of the community (i.e., tolerant, moderately tolerant, moderately intolerant, and intolerant of pollution). Given the long-term reductions in metal concentrations that have occurred in the Spring River and its tributaries, we would expect a recovery in riffle fish species prevalence and diversity, especially in pollution-intolerant species in the previously polluted lower Spring River. Based on riffle fish species long-term responses to pollution reduction, inferences can be made to assess the success of pollution legislation and remediation and whether further regulations and conservation measures need to be initiated. For example, if pollution intolerant species do not recolonize and recover following pollution reduction efforts, then further conservation actions are necessary to protect and conserve these vulnerable species. This highlights the need for further research to investigate the long-term responses of biological indicators to pollution regulations and conservation actions (Alexander and Allan 2007; Whitney et al. 2019).

Methods

Contemporary Data Collection

For our contemporary survey, we sampled 10 sites distributed along the Spring River mainstem in Kansas from the Missouri to Oklahoma borders (Fig. 1). Five sites were positioned above mining pollution (i.e., above the Center Creek confluence), and the other five sites were located below (Fig. 1). Each site was sampled once each year during 2019-2021, excluding the two most downstream sites that were not sampled during 2019 because of high flows. As such, our resulting sample size was 28. We used kick seining to sample riffle fishes, which involved holding a 4.6 m wide X 1.8 m tall seine with 3.2 mm mesh in a stationary position as 1-2 people kicked and disturbed substrate along a 4 m distance downstream towards the seine. The goal was to scare fishes into the seine for individual collection. The seine was held with some slack to create a small bag in which fishes could gather, resulting in an effective sampling width of 4.0 m. As such, each kick-seining effort covered a 16 m^2 area (i.e., 4.0 m seine width x 4.0 m kicking distance = 16 m^2). Captured fishes were carefully placed in buckets after each seine haul, and multiple seine hauls were taken per habitat until an entire habitat had been sampled. After the completion of habitat sampling, we identified each individual fish species and measured their total length in millimeters, then all fishes were safely released back into their respective habitats where we originally collected them. We sampled 2-3 habitats per site, depending on habitat availability within a site.

Historical Datasets

To investigate long-term changes in riffle communities we compared our contemporary riffle fish data to data collected by Edds and Dorlac (1995), Wilkinson and Edds (1996), and Wilkinson and Edds (2001), which were all part of the same study performed during 1993-1995 (Fig. 1). All historical surveys collected community data and relied on similar sampling techniques as our contemporary survey. However, we note that the 1990s surveys expended greater sampling effort in pool habitats compared to our study, collecting many more pool species and individuals using sweep-seining in addition to kick-seining. However, this should not complicate our temporal community comparisons since we only analyzed riffle species during both time periods (see further details below). The 1993-1995 survey sampled 10 sites above and 14 below the Center Creek confluence, resulting in 24 total sites compared to our 28 (i.e., 15 above and 13 below Center Creek).

Data Analysis

We began by classifying all species encountered across both surveys as riffle or pool species using information available in Kansas Fishes (2014) and Hitchman et al. (2018), and we then excluded pool species from further analyses since our research focus was on riffle fishes. Furthermore, although the Sunburst Darter (*Etheostoma mihileze*) was a riffle species, we also excluded it from analyses given its extreme rarity during both time periods (i.e., 2 individuals captured during 1993-1995 and 0 captured during 2019-2021). We then classified each riffle fish species as intolerant, moderately intolerant, moderately tolerant, and tolerant of pollution using classifications from Grabarkiewicz and Davis (2008) and references therein (Table 1). We then calculated species richness (i.e., number of species), occupancy (i.e., number of sites where a species was detected divided by the total number of sites sampled), and relative abundance (i.e., number of individuals captured for a species divided by the total number of individuals captured across all species) of each pollution-tolerance category separately across both datasets. These three metrics served as our community structure response variables in further univariate data analyses.

All statistical analyses were performed in program R (R Core Team 2022). We used a three-way analysis of variance (ANOVA) to examine the interactive effects of time period (historical vs. contemporary), pollution tolerance (four categories), and site position (above versus below metal pollution inputs) on temporal changes in richness, occupancy, and relative abundance of riffle fishes in the Spring River. To satisfy normality assumptions, proportional data (i.e., occupancy and relative abundance) were empirical logit transformed prior to ANOVA using an ε equal to the smallest non-zero occupancy value, or equal to one minus the smallest non-zero relative abundance value (Collett 2002; Warton and Hui 2011). However, all means and 95% confidence intervals were back-transformed from the logit scale prior to visual display in dot plots with error bars. Tukey's post hoc tests were used to determine which treatment combinations differed from one another if the ANOVA found significance ($\alpha = 0.05$). We predicted increasing richness, occupancy, and relative abundance of pollution-intolerant fishes over time, especially in the Spring River below Center Creek.

To determine if overall community structure changed significantly in response to pollution reduction, we used two-way multivariate analysis of variance (MANOVA) to look at the interactive effects of time period and site position on square root transformed relative abundances of individual species. Significant multivariate differences were visualized with biplots and ellipses drawn using non-metric multidimensional scaling (NMDS). The NMDS biplots were created from a Bray-Curtis distance matrix and ellipses were drawn according to the standard deviation of site scores. The MANOVA and NMDS were performed because they allowed us to determine the individual species that were contributing most to overall changes in community structure between the 1990s and the present.

Results

Mining remediation influenced the abundance and distribution of riffle fishes in the Spring River subbasin since the 1990s (Fig. 3). Overall, there was a significant interaction between time period and pollution tolerance for both relative abundance ($F_{3,124}$ = 3.51; P=0.013) and occupancy ($F_{3,124}$ = 2.81; P=0.043) of riffle fishes in the SRS, but the 3-way interaction among time period, tolerance, and site position was not significant (P> 0.06) for either response variable. In both the upper and lower Spring River intolerant and moderately tolerant species increased in occupancy and relative abundance between time periods, tolerant species decreased, and moderately intolerant species showed minimal changes.

In contrast to occupancy and relative abundance, the three-way interaction among time period, pollution tolerance, and site position was significant in describing species richness changes ($F_{3,192}$ = 4.21; P = 0.007; Fig. 4). This analysis revealed that tolerant and moderately tolerant species were more species-poor than intolerant and moderately intolerant species regardless of time period. Furthermore, intolerant and moderately intolerantly species increased in richness over time below pollution inputs, while remaining the same (intolerant) or declining (moderately intolerant) in richness above pollution inputs. Intolerant and moderately intolerant species were historically fewer in the Spring River below metal pollution than they were in the upper Spring River, but their richness increased over time to the extent that the two reaches were similar in richness during the contemporary time period.

Riffle fish community structure in the Spring River significantly differed according to the main effects of time period ($F_{1,48} = 14.6$; $P < 0.001$; Fig. 5) and site position ($F_{1,48} = 2.3$; $P = 0.04$; Fig. 5), but there was no interaction between these factors ($F_{1,48} = 2.2$; $P = 0.06$). The NMDS biplot with ellipses drawn according to time period revealed a shift along the first axis from more positive scores in the 1990s to more negative scores during the 2020s. This shift was largely driven by greater relative abundance during 2019-2021 of several intolerant (especially Neosho Madtom, Gravel Chub, and Banded Darter), moderately intolerant (especially Black Redhorse and Freckled Madtom), and moderately tolerant species (Mimic Shiner), while all tolerant (especially Bullhead Minnow) and a few moderately intolerant species (e.g., Ghost Shiner) were more common during the 1990s. Although statistically significant, differences in community structure between sites above Center Creek versus those below were less pronounced given the greater overlap between the above versus below pollution ellipses. However, the NMDS biplot with ellipses drawn according to site position showed that intolerant and moderately intolerant species were more common in sites located above metal pollution, while tolerant and a few moderately intolerant species (especially Ghost Shiner, River Darter, Brook Silverside, and Ozark Logperch) were more abundant in sites located in the lower Spring River below metal pollution inputs. These multivariate results indicated that nearly all intolerant riffle fishes in the Spring River increased in relative abundance between 1993-1995 to 2019-2021.

Discussion

The implementation of the CWA and SMCRA had a significant effect on relative abundance, occupancy, species richness, and overall community structure of riffle fishes in the SRS. As predicted, pollution intolerant fishes exhibited positive responses (i.e., increases in abundance, occupancy, and species richness) to water quality improvements, especially below historic pollution inputs (i.e., Center Creek, Turkey Creek, and Shoal Creek). However, contrary to our predictions that all fish species would benefit from improving water quality, pollution tolerant species actually became scarcer even while pollution intolerant species increased in prevalence. The shift in riffle fish community structure from pollution tolerant species to pollution intolerant species during the 1990s to 2020s, particularly in the lower Spring River that had been heavily impacted by mining pollution, is likely attributable to improvements in water quality (i.e., lower Pb, Cd, and Zn concentrations). This study provided further evidence that heavy metals depress fish species, particularly riffle fish species that are highly sensitive to heavy metal pollution. These findings have conservation implications, as aside from the Carmine Shiner, all pollution intolerant riffle fish species in the Spring River are imperiled at the state or federal level (Table 1).

Even though many imperiled species responded positively to improving water quality, there were still some species that did not respond to pollution reductions. For instance, the Arkansas Darter is classified as a pollution intolerant species, yet this species was not collected during the 2019-2021 survey. This is surprising because the Arkansas Darter has expanded its range in the SRS (Whitney et al. 2018). Additionally, there were several moderately intolerant species (i.e., Ozark Logperch and River Darter) that were not as prevalent in the contemporary (2019-2021) survey, including the elusive Sunburst Darter, which was not collected at all. All of the moderately intolerant species without SINC classifications had lower relative abundance in our contemporary survey, particularly Brooks Silverside and Ghost Shiner. Since not all imperiled and moderately intolerant species increased in prevalence between time periods, this could be indicative that these fish species are more sensitive to other ongoing pollutants, and in the future, their pollution tolerance classification may need to be reevaluated.

Many studies indicate that harmful chemical pollutants reduce fish diversity, species richness, and abundance (Yount and Niemi 1990; Allert et al. 2009; Mebane et al. 2015 Boroughs et al. 2020), and several studies have examined fish communities before and after pollution reduction. However, few studies have detected fish communities positively responding to mining remediation efforts and resultant water quality improvements. For instance, Whitney et al. (2019) conducted a similar study within the SRS examining pollution tolerance categories of fishes and their responses to water quality improvements following mining remediation and improvements in wastewater treatment resulting from pollution legislation (i.e., CWA and SMCRA), though the results of that study did not detect a positive response to improving water quality in intolerant fishes throughout most of their study area. Although, it is important to recognize in Whitney et al. (2019) that moderately intolerant fishes did positively respond to mining remediation in one stream (i.e., Brush Creek), which previously had a pH that was too low to support fishes. There are, however, several studies that support our results (Yount and Niemi 1990; Jeffree et al. 2001; Fransen 2006; Mebane et al. 2015). Fransen (2006), found that species richness, corrected for habitat size, was significantly lower in sites with higher concentrations of heavy metals. Jeffree et al. (2001) studied fish communities in the Finniss River that had been altered by the Rum Jungle uranium/copper mining, and they found that fish communities reverted to unimpacted stream structure after mining remediation. In a long-term study, Happel and Gallager (2022) attributed increases in fish species richness from 1985 to 2020 in the Chicago Waterways with reductions in wastewater-related contaminants and improvements in water quality. Therefore, significant species recoveries (i.e., in species richness and abundance) following reductions in heavy metals are more apparent in heavily polluted systems and reaches, and species limited to specific habitats (i.e., riffles) are likely more vulnerable to pollution than other species that have adapted to harsher conditions. Therefore, the results of our study are supported by numerous studies in various river systems around the world that were impacted by different pollutants, and for the most part, in all the studies the outcomes were the same: fishes respond positively to pollution reductions.

Future research can build upon the results of our study to address limitations and examine other stressors that may be hindering recolonization and recovery of imperiled SRS fishes. One limitation of this study was the lack of recent water quality data, as the most recent Kansas Department of Health and Environment heavy metal data that is publicly available was collected in 2016 before our contemporary study took place. It remains unclear how heavy metal concentrations have changed since 2016, although we assume that concentrations have continued their long-term decrease. Furthermore, water quality data was only available at a subset of our study sites (Fig. 2), thus less is understood regarding the disparity of heavy metal concentrations among sites within stream segments. Collection of contemporary heavy metal concentrations at a fine spatial resolution could address both of these study limitations. Additionally, heavy metals are just one of several chemical pollutants impacting the SRS, therefore, another avenue for future research includes examining concentrations of other pollutants, especially those of emerging concerning (e.g., endocrine disrupters, microplastics, pharmaceuticals, pesticides), to see if they may explain why not all riffle fish species are recovering from reduced heavy metal concentrations (i.e., Sunburst Darter and Arkansas Darter). These potential research efforts could greatly expand our understanding of the chemical stressors imperiling native fishes of the Spring River.

Improving water quality in the SRS with minimal management intervention has stimulated the recovery of imperiled species, resulting in a rare conservation success story for stream fishes. This study revealed the importance of water quality improvements and the lasting impacts legislation can have on the health of stream ecosystems, particularly on pollution sensitive fish species. Pollution legislation (i.e., CWA and SMCRA) mitigated the side effects of heavy metal pollution in the SRS by improving water quality. Heavy metal remediation had positive effects on riffle fish communities in the Spring River subbasin. Therefore, enacting pollution regulations and practicing proactive conservation strategies, such as habitat restoration and imperiled species propagation and repatriation, is essential for preserving the integrity of streams and conserving pollution sensitive species. However, further monitoring remains necessary to see if this stream fish success story is to be maintained, as increasing concentrations of pollutants of emerging concern from nearby cities in the Spring River subbasin (e.g., Carthage, Joplin, and Pittsburg; Fig. 1) could reduce and potentially reverse the recovery of imperiled fish species.

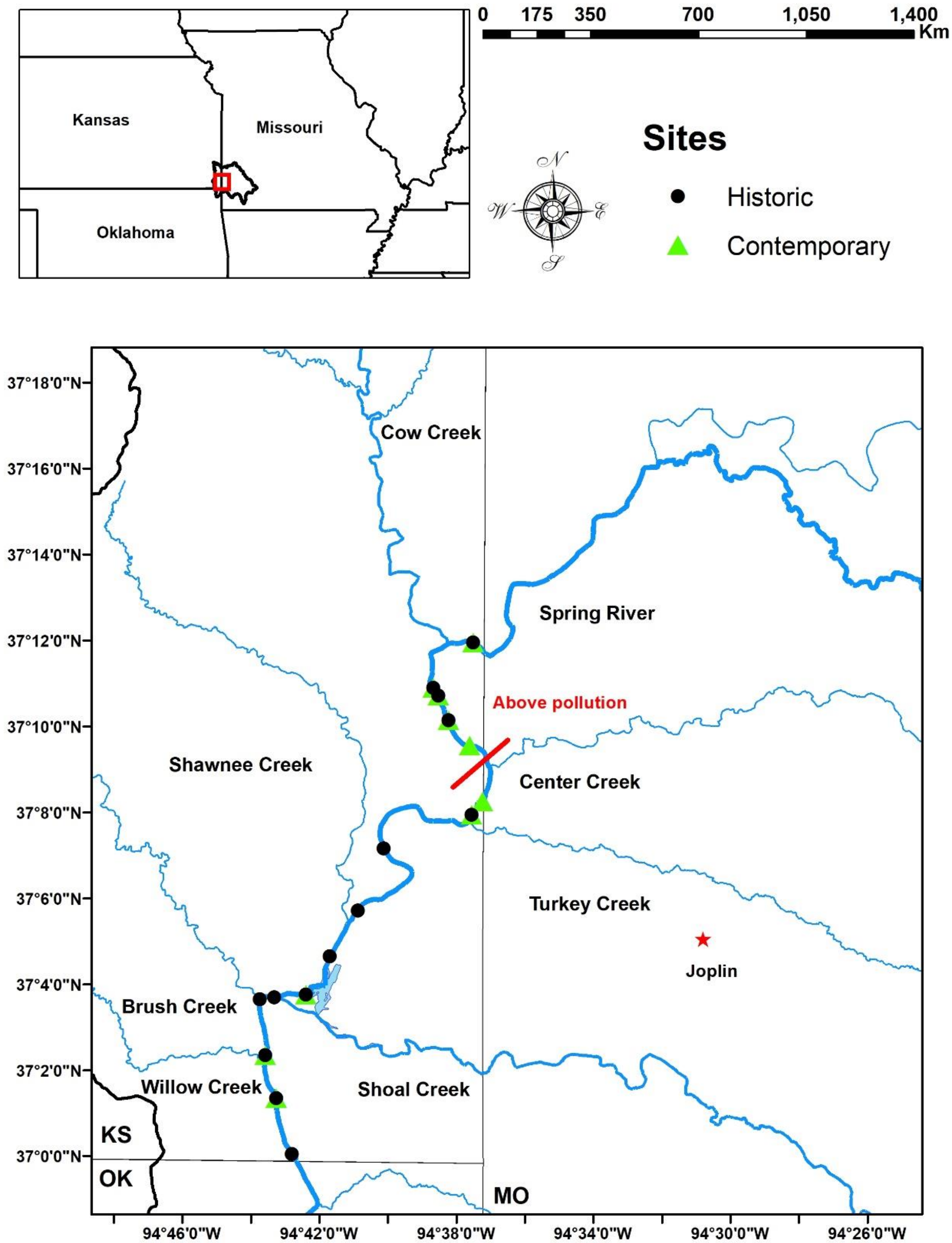


Figure 1. The Spring River subbasin in the broad extent and locations of historic (1993-1995) and contemporary sites (2019-2021) in relation to pollution inputs in the fine extent.

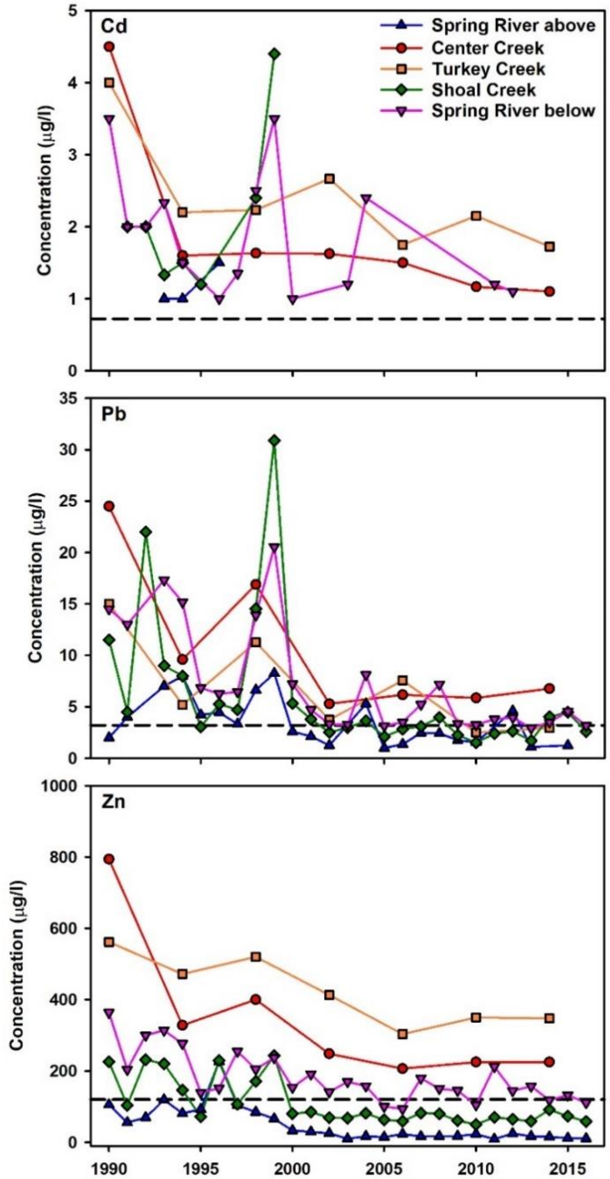


Figure 2. Trends in heavy metals (i.e., cadmium, lead, and zinc) concentrations from the 1990 to 2016 in the Spring River subbasin. Data were collected by the Kansas Department of Health and Environment. The dashed lines represent the chronic concentration set by the Environmental Protection Agency, wherein long-term concentrations above these values would be harmful to aquatic life.

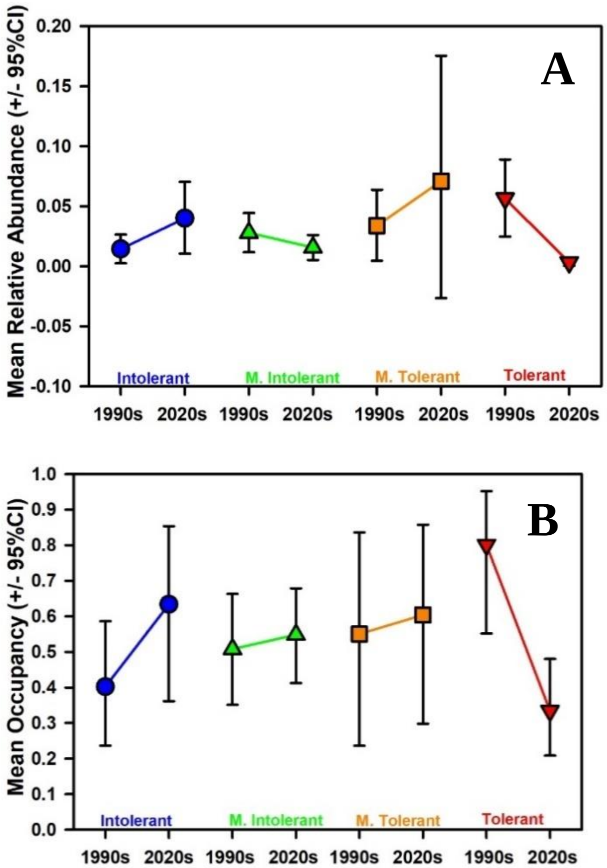


Figure 3. **A)** Mean relative abundance and **B)** occupancy across all four categories of pollution tolerances for riffle fishes in the Spring River subbasin before (1993-1995) and after (2019-2021) reduced heavy metal concentrations.

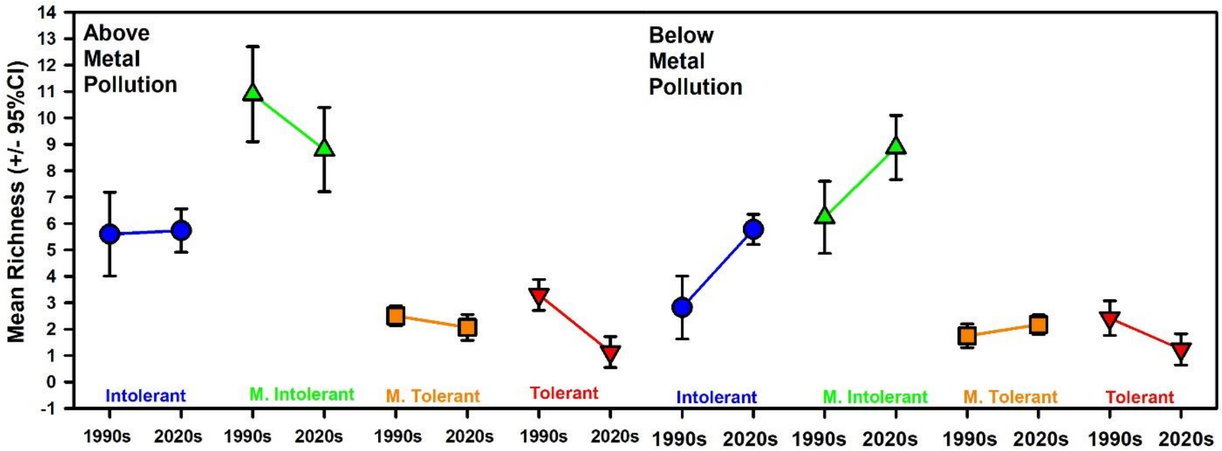


Figure 4. Species richness across all four categories of pollution tolerances for riffle fishes in the Spring River subbasin before (1993-1995) and after (2019-2021) mining remediation with regards to their position above and below mining pollution.

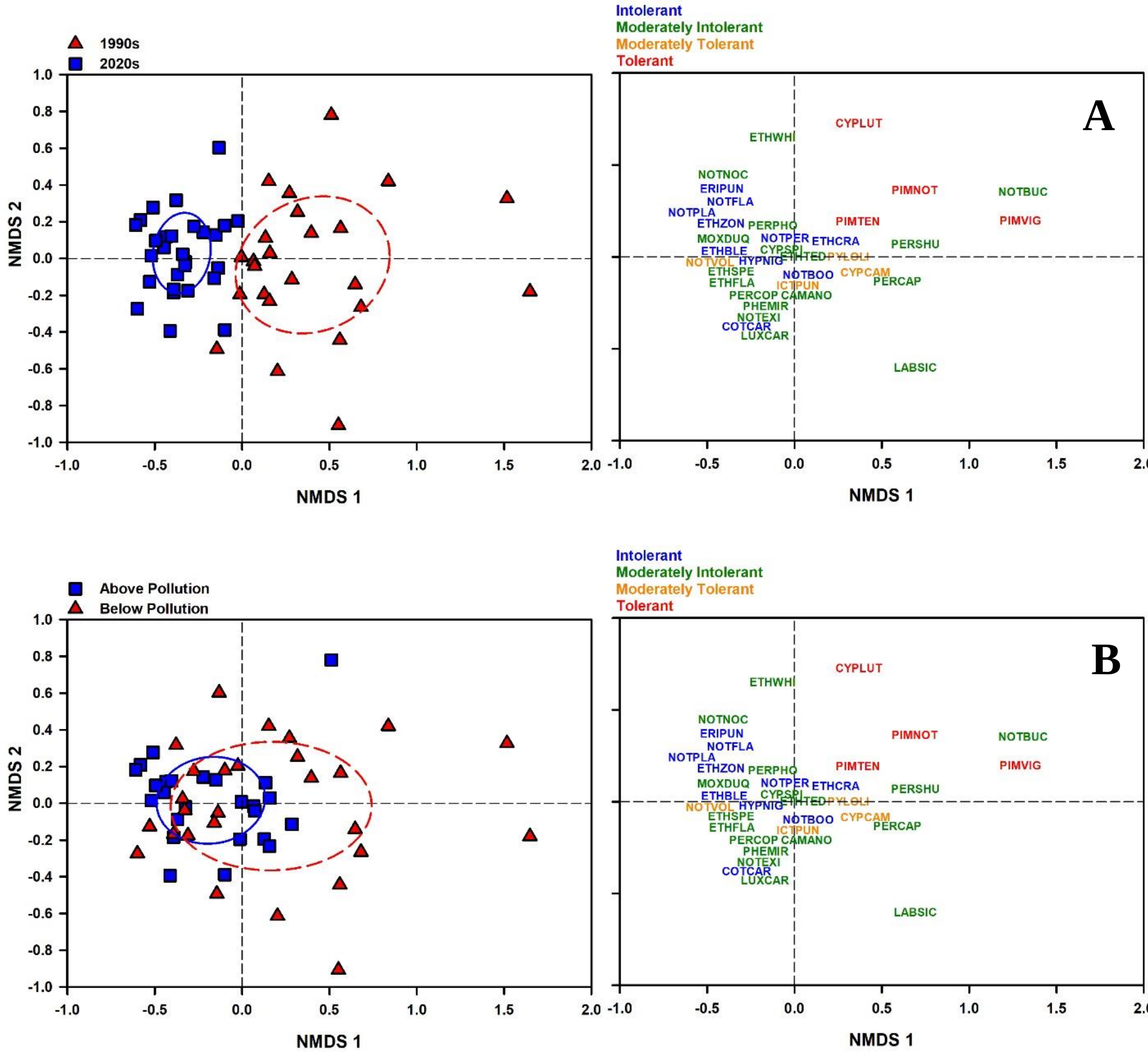


Figure 5. **A)** Non-metric multidimensional scaling (NMDS) biplot comparing 1990's versus 2020's riffle fish community structure in the Spring River of Kansas. **B)** NMDS biplot for riffle fish community structure above versus below mining pollution. See Table 1 for species code explanations.

Table 1. Pollution tolerance classifications, species of greatest conservation need (SGCN) tiers, and number of individuals collected for riffle fishes in the Spring River subbasin during 1993-1995 and 2019-2021. Lower SGCN tiers indicate greater conservation need.

Common name	Scientific name	Species Code	Pollution tolerance	SGCN	1993-1995	2019-2021
Arkansas Darter	*Etheostoma cragini*	ETHCRA	Intolerant	1	9	0
Banded Darter	*Etheostoma zonale*	ETHZON	Intolerant	2	260	2005
Banded Sculpin	*Cottus carolinae*	COTCAR	Intolerant	2	1	3
Bigeye Shiner	*Notropis boops*	NOTBOO	Intolerant	2	3	4
Carmine Shiner	*Notropis percobromus*	NOTPER	Intolerant	None	659	1149
Fantail Darter	*Etheostoma flabellare*	ETHFLA	Intolerant	2	67	49
Gravel Chub	*Erimystax x-punctatus*	ERIPUN	Intolerant	2	126	1825
Greenside Darter	*Etheostoma blennioides*	ETHBLE	Intolerant	2	60	129
Neosho Madtom	*Noturus placidus*	NOTPLA	Intolerant	1	15	179
Northern Hog Sucker	*Hypentelium nigricans*	HYPNIG	Intolerant	2	28	64
Stonecat	*Noturus flavus*	NOTFLA	Intolerant	2	23	74
Black Redhorse	*Moxostoma duquesnei*	MOXDUQ	Moderately intolerant	2	4	13
Brook Silverside	*Labidesthes sicculus*	LABSIC	Moderately intolerant	None	924	56
Cardinal Shiner	*Luxilus cardinalis*	LUXCAR	Moderately intolerant	2	1163	1645
Central Stoneroller	*Campostoma anomalum*	CAMANO	Moderately intolerant	None	298	239
Channel Darter	*Percina copelandi*	PERCOP	Moderately intolerant	2	139	418
Freckled Madtom	*Noturus nocturnus*	NOTNOC	Moderately intolerant	2	0	8
Ghost Shiner	*Notropis buchanani*	NOTBUC	Moderately intolerant	None	200	26
Highland Darter	*Etheostoma teddyroosevelt*	ETHTED	Moderately intolerant	2	39	40
Orangethroat Darter	*Etheostoma spectabile*	ETHSPE	Moderately intolerant	2	263	618
Ozark Logperch	*Percina caprodes*	PERCAP	Moderately intolerant	2	163	62
Redfin Darter	*Etheostoma whipplei*	ETHWHI	Moderately intolerant	2	8	17
River Darter	*Percina shumardi*	PERSHU	Moderately intolerant	2	12	1
Slender Madtom	*Noturus exilis*	NOTEXI	Moderately intolerant	2	133	68
Slenderhead Darter	*Percina phoxocephala*	PERPHO	Moderately intolerant	2	144	170
Spotfin Shiner	*Cyprinella spiloptera*	CYPSPI	Moderately intolerant	2	131	44
Suckermouth Minnow	*Phenacobius mirabilis*	PHEMIR	Moderately intolerant	None	198	100
Sunburst Darter	*Etheostoma mihileze*	ETHMIH	Moderately intolerant	2	2	0
Bluntface Shiner	*Cyprinella camura*	CYPCAM	Moderately tolerant	None	685	18
Channel Catfish	*Ictalurus punctatus*	ICTPUN	Moderately tolerant	None	325	316
Flathead Catfish	*Pylodictis olivaris*	PYLOLI	Moderately tolerant	None	4	12
Mimic Shiner	*Notropis volucellus*	NOTVOL	Moderately tolerant	None	90	3631
Bluntnose Minnow	*Pimephales notatus*	PIMNOT	Tolerant	None	816	54
Bullhead Minnow	*Pimephales vigilax*	PIMVIG	Tolerant	None	227	17
Red Shiner	*Cyprinella lutrensis*	CYPLUT	Tolerant	None	564	97
Slim Minnow	*Pimephales tenellus*	PIMTEN	Tolerant	None	231	10
Grand Total					**8014**	**13161**

Chapter II

CONTEMPORARY EXTENT OF THE BLACKSPOTTED TOPMINNOW INVASION AND FREQUENCY OF HYBRIDIZATION WITH NATIVE BLACKSTRIPE TOPMINNOW IN THE SPRING RIVER SUBBASIN

Introduction

Blackspotted (*Fundulus olivaceus*) and Blackstripe Topminnow (*Fundulus notatus*) are ecologically similar sister species that have ranges extending throughout much of the Mississippi River drainage and coastal drainages of the Gulf of Mexico (Schaefer 2014a; 2014b). Previous studies have suggested that these two species are reproductively compatible despite cytogenetic differences and will hybridize in syntopic populations (Duvernell et al. 2007). The diet of Blackspotted Topminnow is similar to Blackstripe Topminnow in that both species feed on terrestrial arthropods, aquatic invertebrates, and primary producers (e.g., diatoms and duckweed) near surface waters, resulting in the potential for competition between the species when they co-occur (Thomerson and Woolridge 1970; Champagne 2011). However, between-species differences in habitat preferences and tolerances may help limit competition via niche segregation, as Blackspotted Topminnow tend to prefer clearer waters and are less tolerant of poor water quality (e.g., hypoxia), whereas Blackstripe Topminnow can tolerate more turbid waters with poorer quality.

Blackspotted Topminnow are typically identified based on the presence of numerous well-defined black spots on their dorsolateral surface, which are faint or absent in Blackstripe Topminnow (Fig. 6). However, the spot phenotype in these species is plastic and depends on water clarity (Schaefer et al. 2012). In turbid waters Blackspotted Topminnow may not exhibit spots, and in clear water Blackstripe Topminnow may develop spots. Furthermore, in Blackspotted Topminnow the spot phenotype is a sexually dimorphic trait that is more common in mature males and is less prevalent in females (Schaefer et al. 2012; Steffensmeier et al. 2020). As such, Blackstripe and Blackspotted Topminnow are notoriously difficult to distinguish from one another given their similar appearances and phenotypic convergence in homogeneous environmental conditions, and there is no way to visually distinguish between hybrids and pure parental species.

Although the Blackspotted Topminnow is one of the most widely distributed *Fundulus* species in the United States (Thomerson and Woolridge 1970; Holcroft 2004), Blackspotted Topminnow is not native to the Spring River subbasin (SRS) of southeastern Kansas, southwestern Missouri, and northeastern Oklahoma (Pflieger 1997; King et al. 2021b). Originally suspected to have been a bait-bucket release, Blackspotted Topminnow was introduced to Shoal Creek in Missouri by 1990 and since that time it has spread into Shoal Creek of Kansas and the Spring River below Empire Lake in Kansas and Oklahoma (Pflieger 1997; Wilkinson and Edds 2001; King et al. 2021b). The first record of Blackspotted Topminnow in Kansas came in 2000 from Shoal Creek in

Schermerhorn Park, Galena, with the species being collected there again in 2002 (Holcroft 2004). Later, in 2008-2010, Duvernell and Schaefer (2014) collected fin clips for genetic analysis from Blackspotted and Blackstripe Topminnow from Shoal Creek and the Spring River below Empire Lake, although they did not sample the Spring River above Empire Lake. In contrast, King et al. (2021b) surveyed for Blackspotted Topminnow in the SRS above Empire Lake, but their methodology was based on visual identification and did not include genetic analysis.

The SRS is divided into three physiographic regions in Kansas: Osage Cuestas, Cherokee Lowlands, and the Ozark Plateau (Fig. 7; Aber and Aber 2009). The headwaters of First and Second Cow Creeks are in the Osage Cuestas, while the rest of the Cow Creek watershed is in the Cherokee Lowlands. To the south, the Spring River mainstem curves into the Cherokee Lowlands from Missouri and flows downstream into the Ozark Plateau. The physiographic regions in the SRS have distinctly unique physicochemical habitat characteristics which may influence the invasion success of Blackspotted Topminnow given their habitat preferences. For instance, the Spring River and its Ozarkian tributaries tend to have clear water, coarse substrate, high velocity, and perennial flow, whereas non-Ozarkian tributaries of the Spring River in the Cherokee Lowlands and Osage Cuestas are generally more turbid, siltier, slower flowing, and intermittent (Davis and Schumacher 1992; Wilkinson and Edds 2001). As such, Blackspotted Topminnow invasion success may be greater in the Ozark Plateau compared to the Cherokee Lowlands and Osage Cuestas, given physicochemical conditions in the Ozark Plateau align with the Blackspotted Topminnow's niche preferences.

Within the SRS, nonnative Blackspotted Topminnow may be having negative interactions with native Blackstripe Topminnow. For instance, Blackspotted Topminnow are known for hybridizing with Blackstripe Topminnow (Duvernell and Schaefer 2014; Schaefer 2014 b) in the SRS. Duvernell and Schaefer (2014) found that individuals from Shoal Creek were mostly pure Blackspotted Topminnow, while individuals in the Spring River directly below Empire Lake were a nearly equal mixture of pure strain Blackspotted Topminnow, pure strain Blackstripe Topminnow, and hybrid individuals. Furthermore, the SRS exhibited one of the highest rates of Blackspotted-Blackstripe Topminnow hybridization across the 10 drainages examined in their study. However, the frequency and distribution of *Fundulus* spp. hybridization in the entire SRS has yet to be quantified since it has not been investigated in the Spring River above Empire Lake. There is evidence that suggested Blackspotted Topminnow are displacing Blackstripe Topminnow in portions of the SRS where they have invaded (King et al. 2021b). Hybridization in conjunction with competition from Blackspotted Topminnow may be causing imperilment of Blackstripe Topminnow in the SRS. However, a widespread survey of the SRS in KS during 2017-2020 did not detect Blackspotted Topminnow in the Spring River above Empire Lake; therefore, Empire Lake may be serving as a dispersal barrier preventing further invasion of Blackspotted Topminnow in KS (King et al. 2021b; Fig. 7). Though, the 2017-2020 survey not detecting Blackspotted Topminnow above Empire Lake could be attributed to misidentification (i.e., false absence) rather than the species being truly absent from this reach given how difficult it is to distinguish these species using visual cues alone. Since the Blackspotted Topminnow invasion is ongoing and impacting native Blackstripe Topminnow prevalence, the need for continual monitoring using traditional sampling methods (i.e., dipnetting, seining) combined with genetic analyses are necessary.

The objectives of this research are to use genetic techniques in the SRS to 1) investigate the contemporary extent of the Blackspotted Topminnow invasion, 2) quantify the frequency of Blackspotted-Blackstripe Topminnow hybridization, 3) examine water clarity in relation to Blackspotted Topminnow and Blackstripe Topminnow distributions, and 4) assess whether Empire Lake acts as a dispersal barrier to the further spread Blackspotted Topminnow. The overall purpose of this research is to gain a better understanding of this nonnative introduction and its impacts on the native Blackstripe Topminnow, and to assess whether a small impoundment (i.e., Empire Lake) acts as a dispersal barrier preventing further invasion upstream. The invasion of Blackspotted Topminnow is unique to the SRS. We could not locate any other documented introductions of Blackspotted Topminnow outside of the SRS since Blackspotted Topminnow is normally sympatric with Blackstripe Topminnow as a native species (Duvernell and Schaefer 2014; Duvernell et al. 2019). This research will help inform the management of nonnative species and native species conservation in the SRS, while also contributing to the field of fish ecology by examining how a dispersal barrier (i.e., Empire Lake) impacts the outcome of an invasion.

Methods

Study Site Selection and Sample Collection

To sample for Blackspotted and Blackstripe Topminnow, we used a combination of seining and dipnetting with the goal of collecting thirty individuals from seven sample sites strategically positioned around Empire Lake during May-October 2021. We sampled along the margins of pools since Blackspotted and Blackstripe Topminnow are generally found in pairs or small groups near thick stands of water willow in low-velocity habitats (Pflieger 1997). Sites sampled included: 1) Second Cow Creek near Girard, KS, 2) the Spring River at the Spring River Wildlife Area just downstream from the Cow Creek confluence, 3) the Spring River directly above Empire Lake at the Highway 66 crossing, 4) the Spring River directly below Empire Lake, 5) Shoal Creek directly above Empire Lake, 6) Shoal Creek at the KS-MO Border, and 7) the Spring River at Baxter Springs (Fig. 7). The Second Cow Creek site was in the Osage Cuestas, whereas all other sites were located in the Ozark Plateau. Captured individuals were preserved in 95% ethanol for later genetic analyses.

After collecting *Fundulus* spp. individuals from sites, we measured turbidity in nephelometric turbidity units (NTUs) by collecting a sample of stream water for later laboratory analyses using a Hach 2100P turbidimeter. Furthermore, since water clarity is strongly influenced by upstream agricultural land use (i.e., more upstream agricultural land use results in greater turbidity), we also quantified the percent upstream watershed area that was agricultural for each study site using the 2011 National Land Cover Dataset. We reasoned that upstream agricultural land use would do a better job of quantifying long-term water clarity at our sites compared to our snapshot turbidity measurements given how much water clarity can vary over time based on recent precipitation, surface runoff, and discharge (Chen and Chang 2019).

Genetic identification

Body tissues for genetic analysis were collected by cutting 1 cm^2 fin clips from the caudal and anal fins, then the clippings were transferred into Eppendorf tubes. We labeled the fish specimens according to their collection location and date, and we assigned numbers to each individual and stored them in scintillation vials filled with 95% ethanol.

We genetically identified individuals using a restriction fragment length polymorphism (RFLP) assay. First, we crushed the fin clips in their labeled Eppendorf tubes with a pestle and added proteinase K and AL buffer before we incubated the solution at 56 °C for 10 min. All the centrifuge steps were performed at room temperature (25 °C). The total DNA was extracted from preserved fin tissues using Qiagen DNeasy Blood and Tissue Kits with mini extraction columns. Following the DNA extraction, we diluted the extracted DNA to 50-100 ng. We amplified extracted DNA at the glutathione peroxidase (GPX) and cold-inducible RNA binding protein (CIRP) nuclear genetic loci, as single nucleotide polymorphisms (SNIPs) at these loci can differentiate Blackspotted from Blackstripe Topminnow (Table 2). Targeted DNA segments were amplified using polymerase chain reaction (PCR) following a combination and minor modification of methodologies from Duvernell and Schaefer (2014) and Steffensmeier et al. (2020). Specifically, 20 µl of PCR reaction included 2 µl of 50-100 ng diluted template DNA, 10 µL of 2X Thermo Scientific Master mix with Taq polymerase, 0.5 µL (200 pmole) each forward and reverse primer, and 7 µL of distilled water. The BioRad C1000 Touch thermal cycler conditions included an initial denaturing step of 95°C for 5 min, thirty-five 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. A handful of PCR reactions were analyzed by 1% agarose gel electrophoresis to confirm the amplicon length (i.e., CIRP 740-800 bp and GPX 550-600 bp; Appendix A). The *cyt*-B gene was used as a positive control. Aliquots of the amplified PCR product were digested with an appropriate restriction enzyme, including *Hae*III for GPX and *Alu*I for CIRP (Table 2) using CutSmart Buffer in a 20 µl digestion reaction (incubate at 37°C for 1 hour followed by inactivation at 80°C for 20 min). Lastly, we electrophoresed the digested DNA on a 1.5% agarose gel in 1X TAE buffer. Based on the banding patterns produced from the PCR-RFLP technique, we classified individuals as pure strain Blackstripe Topminnow, pure strain Blackspotted Topminnow, F_1 hybrid, Blackstripe Topminnow backcross, and Blackspotted Topminnow backcross (Table 3; Appendix B; Appendix C).

Statistical analysis

To assess whether Empire Lake is acting as a dispersal barrier for Blackspotted Topminnow and hybrids, we determined if the proportion of individuals that are pure strain Blackstripe Topminnow, pure strain Blackspotted Topminnow, hybrid, or backcrossed differed among our study sites using a χ^2 test of independence. We also used simple logistic regression to see if the proportions of individuals in the aforementioned categories varied according to the percentage of the upstream watershed area of sample sites that was in agricultural land use. The significance of logistic regressions was evaluated using a Wald χ^2 test. We also examined the effects of turbidity on the proportions of individuals; however, turbidity varies throughout the year and without long term averages of turbidity data, it was not a reliable predictor variable in this study. All statistical analyses were performed in program R (R Core Team 2022) using an $\alpha = 0.05$.

Results

We collected a total of 169 individuals for genetic analyses, including 16 from Second Cow Creek, 32 from Shoal Creek at the MO border, 30 from near the Shoal Creek-Empire Lake confluence, 5 from the Spring River at the Spring River Wildlife Area, 31 at the Spring River above Empire Lake, 35 from the Spring River below Empire Lake, and 20 from the Spring River at Baxter Springs. We found Blackspotted Topminnow in 4/7 (57%) sites we sampled, including collections from both Shoal Creek sites, the Spring River directly below Empire Lake, and the Spring River at Baxter Springs (Fig. 8; Table 4). Blackspotted Topminnow were most prevalent in the Shoal Creek at the KS-MO border (78%), but only composed 7% of individuals collected in the Shoal Creek-Empire Lake confluence (Fig. 8; Table 4). Blackspotted Topminnow were not present in Second Cow Creek, or at the Spring River above Empire Lake or at the Spring River Wildlife Area. Only Blackstripe Topminnow were collected at Second Cow Creek and both Spring River sites located above Empire Lake and composed 90% of individuals at Baxter Springs. The Spring River directly below Empire Lake was a hotspot for Blackstripe-Blackspotted Topminnow hybridization with a ratio of 40% pure strain Blackstripe Topminnow, 20% pure strain Blackspotted Topminnow, 34% hybrids, and 6% backcrossed individuals (Fig. 8; Table 4). Hybrids were also present in Shoal Creek at both the Empire Lake confluence (70%) and near the KS-MO border (13%). These three sites where hybrids were most prevalent were the only sites where we detected backcrossing among hybrids and Blackspotted Topminnow, and there were no Blackstripe Topminnow backcrossed individuals detected in this study. Backcrossed Blackspotted Topminnow were rare at these three sites though, never comprising more than 9% of individuals examined.

The proportions of pure strain Blackspotted and Blackstripe Topminnow, hybrids, and backcrosses significantly differed among sites ($\chi^2 = 173.56$; df = 18; $P < 0.001$; Fig. 8). This variation was explained by the percentage of agriculture in the upstream watershed area (Fig. 9), as the proportion of pure strain Blackspotted Topminnow ($\chi^2 = 23.045$; df = 1; $P < 0.001$) and hybrid individuals ($\chi^2 = 16.933$; df = 1; $P <0.001$) decreased with greater upstream agricultural land use, whereas the proportion of Blackstripe Topminnow increased ($\chi^2 = 48.793$; df = 1; $P < 0.001$). In contrast, the percent upstream agricultural land use was not related to proportions of backcrossed individuals ($\chi^2 = 3.0832$; df = 1; p-value =0.0791). Among study streams Second Cow Creek had the greatest percentage of agricultural land use (~90%), Shoal Creek had the lowest (~56%), and the Spring River was intermediate (~74% agricultural land use) (Table 5; Fig. 10).

Discussion

This research expanded our knowledge on the distribution and prevalence of nonnative Blackspotted Topminnow in the SRS. Our results were consistent with our initial predictions, in that Shoal Creek near the KS-MO border had the highest prevalence of Blackspotted Topminnow in the SRS. This was not surprising considering Blackspotted Topminnow were first introduced to Shoal Creek in Missouri the 1990s, the low turbidity of this stream resulting from less agriculture in its watershed, and previous genetic findings by Duvernell and Schaefer (2014) that found individuals from Shoal Creek were mostly pure strain Blackspotted Topminnow. However, previous genetic surveys did not sample near the Shoal Creek-Empire Lake confluence. As such, our findings provided new insight since we found Blackstripe Topminnow continued to coexist and hybridize with Blackspotted Topminnow in Shoal Creek near the Empire Lake confluence. This indicated that Blackspotted Topminnow have not fully displaced Blackstripe Topminnow from Shoal Creek. However, in time Blackspotted Topminnow may completely displace Blackstripe Topminnow from Shoal Creek if competitive exclusion or the hybridization frequency between these two *Fundulus* species increases. For instance, within a 40-year span, Blackspotted Topminnow completely displaced Blackstripe Topminnow in the Bourbeuse and upper Meramec Rivers that were historically exclusively inhabited by Blackstripe Topminnow (Steffensmeier et al. 2020). The Shoal Creek Empire Lake confluence did have the highest hybridization rate (i.e., 70% hybrid individuals) among our study sites, which was double the hybridization rate in the Spring River directly below Empire Lake (34%) that had been previously identified by Duvernell and Schaefer (2014). The contact and hybridization zone at the Shoal Creek-Empire Lake confluence was previously unknown, and as such provides novel understanding regarding the Blackspotted Topminnow invasion of the SRS.

Our research supports findings from King et al. (2021b) and suggested through genetic identification of *Fundulus* individuals that Empire Lake may be acting as a dispersal barrier against further Blackspotted Topminnow invasion. Our study provided evidence that Blackspotted Topminnow are not currently present in the Spring River above Empire Lake, which was also an important finding given previous genetic surveys had not been conducted in this segment of the Spring River. However, variation in turbidity resulting from agricultural land use alone or in combination with a dispersal barrier (i.e., Empire Lake) could also explain our patterns. For instance, Blackstripe Topminnow were dominant in streams above Empire Lake that were more vulnerable to soil erosion and sediment runoff from agricultural land use, which would result in higher turbidity that could limit the distribution of Blackspotted Topminnow in these streams. In contrast, Blackspotted Topminnow were more prevalent in Shoal Creek, which had low turbidity and percent agricultural area as a result of more forested land in its Ozarkian watershed. Lastly, the prevalence of Blackspotted Topminnow and hybrid individuals in the Spring River directly below Empire Lake could be explained by low turbidity as well, since suspended sediment deposition in low-velocity reservoirs results in river outflows below dams having characteristically clear water.

Given their clear water, other Ozarkian tributaries of the Spring River where Blackspotted Topminnow are currently unknown could be prone to invasion by this introduced species. These streams include Center Creek, Turkey Creek and Five-Mile Creek. These streams have not been previously surveyed for Blackspotted Topminnow using genetic identification, thus future studies should perform these analyses to verify that Blackspotted Topminnow are truly absent from these streams. If so, public outreach and education could help prevent Blackspotted Topminnow introductions to these streams via bait bucket releases, which previously occurred in Shoal Creek (Pflieger 1997). Furthermore, frequent and continued monitoring could help identify an introduced population should one be discovered. These conservation measures could help ensure introduced Blackspotted Topminnow do not become more prevalent in the SRS over time.

Figure 6**.** Lateral view of Blackstripe Topminnow (left; *Fundulus notatus*) and Blackspotted Topminnow (right; *Fundulus olivaceus*) collected from the Spring River subbasin of Kansas.

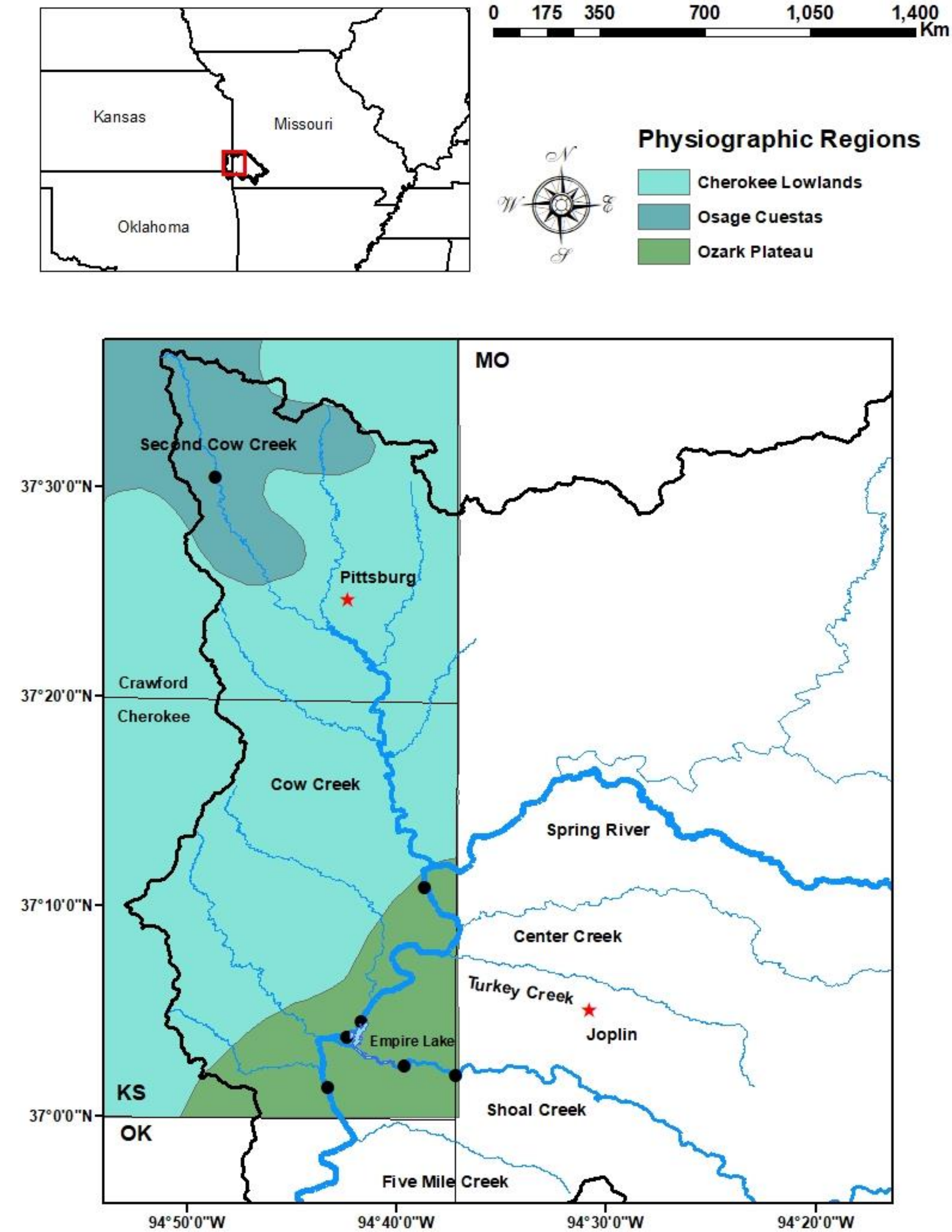


Figure 7. The Spring River subbasin in the broad extent and *Fundulus* spp. collection locations in the fine extent.

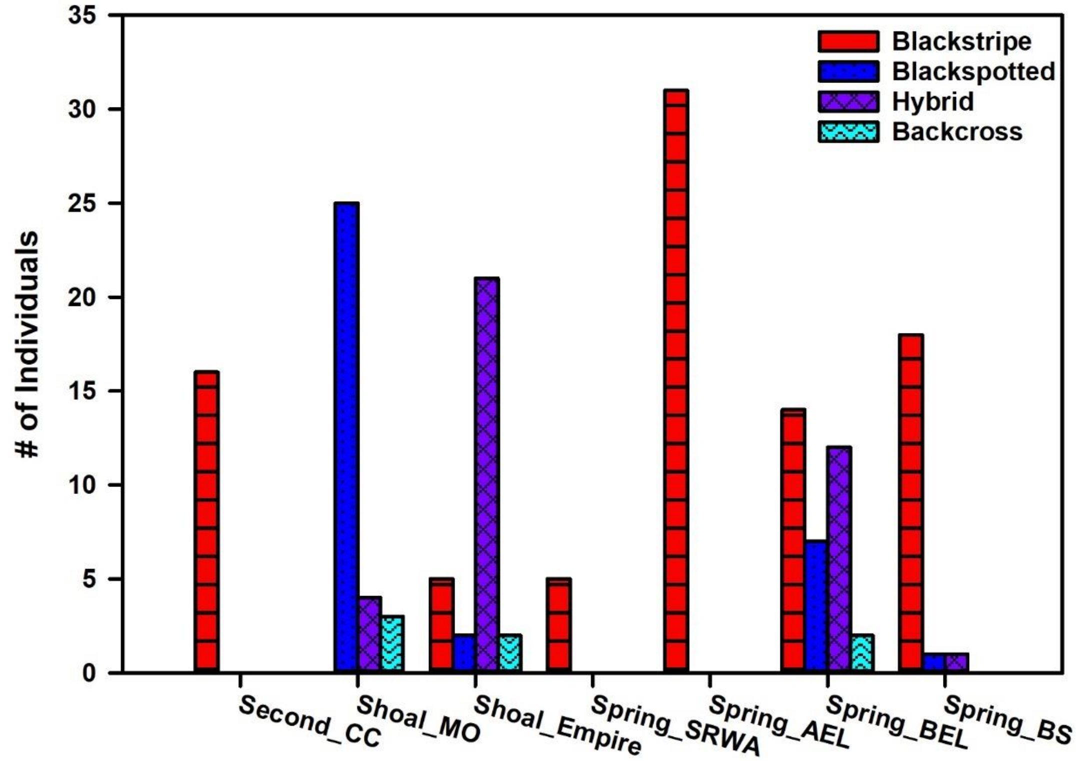


Figure 8. Number of individuals (i.e., pure strain Blackspotted Topminnow, pure strain Blackstripe Topminnow, hybrid, and backcross) collected at the 7 sites in the SRS.

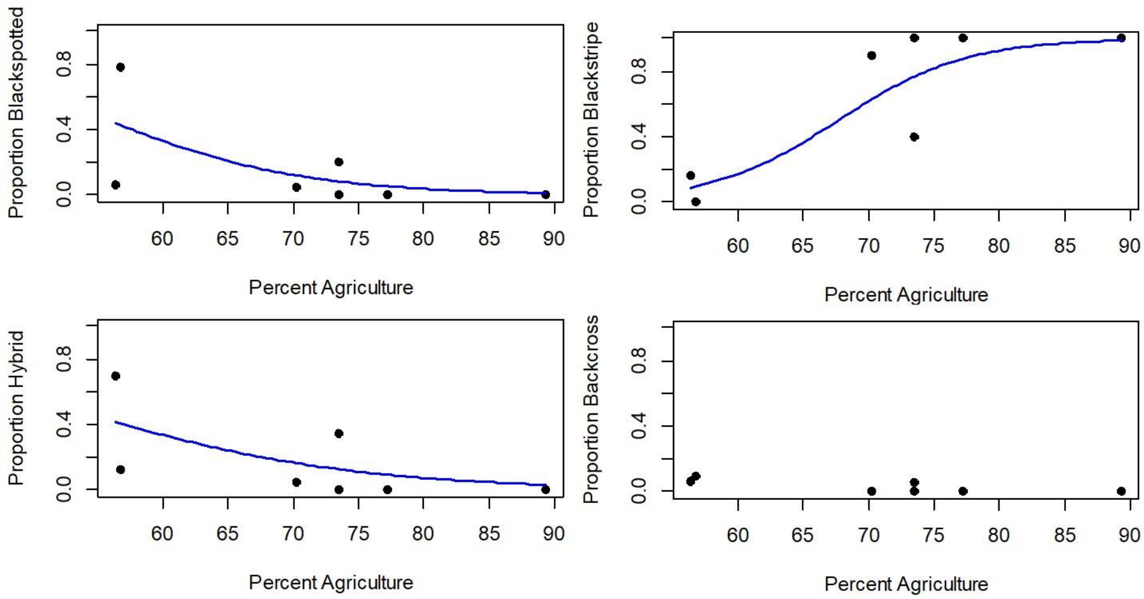


Figure 9. Proportions of individuals (pure strain Blackspotted Topminnow, pure strain Blackstripe Topminnow, hybrid, and backcross individuals) according to the percent upstream agricultural land use for each collection site.

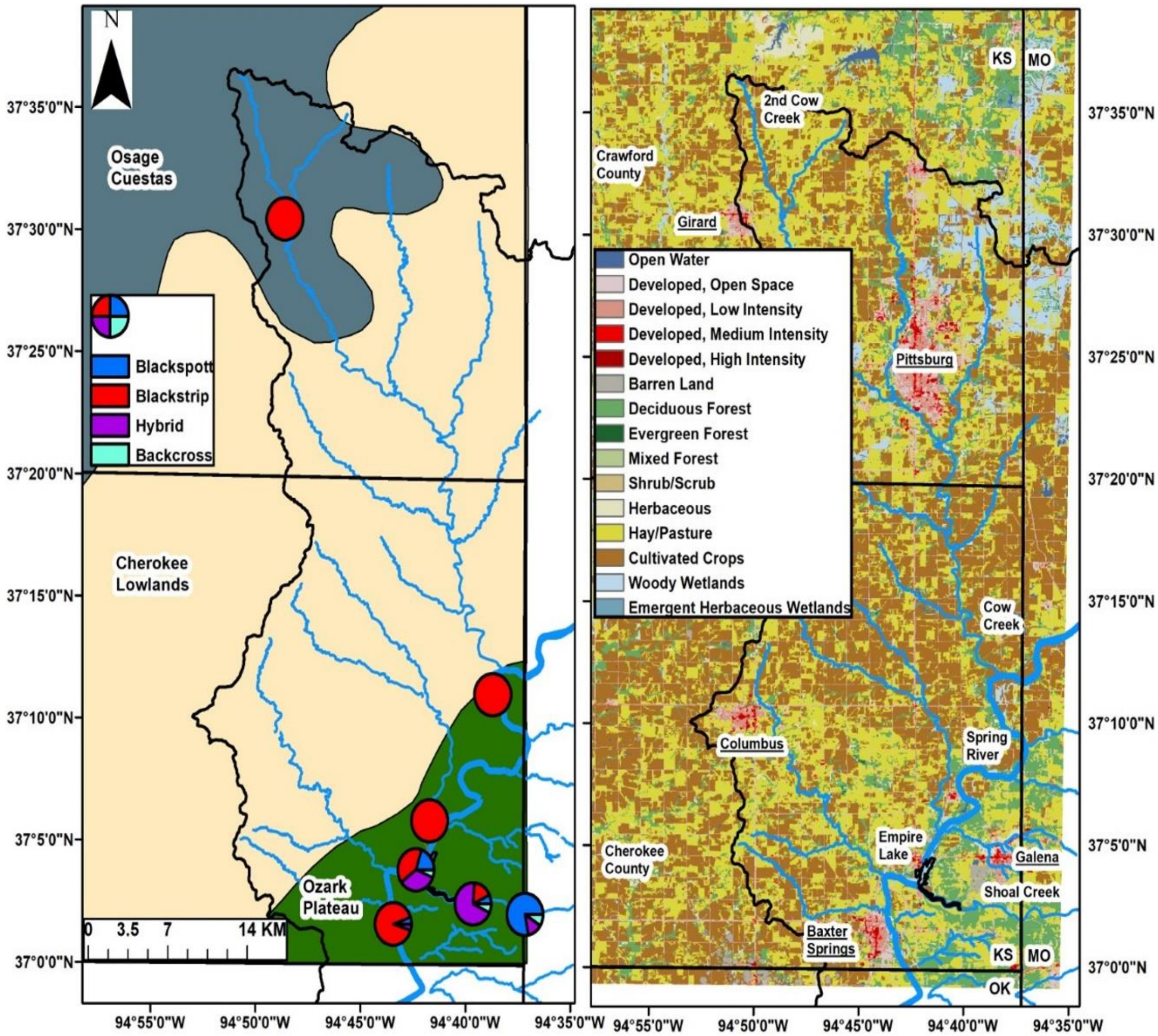


Figure 10. Sites with proportions of *Fundulus* species (i.e., pure strain Blackstripe Topminnow, pure strain Blackspotted Topminnow, hybrids, and backcrosses) plotted according to physiographic regions, with land use and land cover in the SRS of Kansas also indicated.

Table 2. Forward and reverse oligonucleotide primers that target the loci of interest for *F. notatus* and *F. olivaceus*, as well as the resGtriction enzymes that will cut target loci for a particular species to reveal diagnostic single nucleotide polymorphisms (Schaefer et al. 2012).

Locus	Oligonucleotide (5`—3`)	Restriction enzyme	Gene length	*F. notatus*	*F. olivaceus*
CIRP	Forward GCTTCGAGACCAACGAAGAC Reverse CGTCACGATACGATCCAGAG	*Alu*I	740-800	Cut	Uncut
GPX	Forward AGGTGAGGAAACCCACCTTT Reverse TAGCGGCCTCTCTCATGTTT	*Hae*III	550-600	Uncut	Cut

Table 3. Expected results from the PCR-RFLP assay.

CIRP Allele 1	CIRP Allele 2	GPX Allele 1	GPX Allele 2	Classification
Cut	Cut	Uncut	Uncut	Pure Strain FUNNOT
Uncut	Uncut	Cut	Cut	Pure Strain FUNOLI
Uncut	Cut	Uncut	Cut	F_1 Hybrid
Cut	Cut	Uncut	Cut	Backcross FUNNOT
Uncut	Uncut	Cut	Uncut	Backcross FUNOLI

Table 4. Numbers and percentages of pure strain Blackstripe Topminnow (FUNNOT), pure strain Blackspotted Topminnow (FUNOLI), hybrids, backcrossed Blackstripe Topminnow, and backcrossed Blackspotted Topminnow individuals collected at each site.

Sites	FUNNOT	FUNOLI	Hybrids	Backcross FUNOLI
Second Cow Creek	16/16 (100%)			
Spring River Wildlife Area	5/5 (100%)			
Spring River Above Empire Lake	31/31 (100%)			
Shoal Creek near MO border		25/32 (78%)	4/32 (13%)	3/32 (9.4%)
Shoal Creek at Empire Lake confluence	5/30 (17%)	2/30 (6.7%)	21/30 (70%)	2/30 (6.7%)
Spring River below Empire Lake	14/35 (40%)	7/35 (20%)	12/35 (34%)	2/35 (5.7%)
Spring River at Baxter Springs	18/20 (90%)	1/20 (5%)	1/20 (5%)	

Table 5. Water quality (i.e., turbidity and percent upstream agricultural land use) of *Fundulus* collection sites. NTU = nephalometric turbidity units.

Site	Turbidity (NTU)	Percent Ag
Second Cow Creek	2.73	89.38
Spring River Wildlife Area	20.475	77.17
Spring River Above Empire Lake	11.19	73.45
Spring River Below Empire Lake	4.875	73.48
Spring River at Baxter Springs	6.31	70.25
Shoal Creek near KS/MO border	3.025	56.77
Shoal Creek near Empire Lake confluence	2.965	56.39

REFERENCES

Aber, J. S. and Aber, S. W. 2009. Kansas physiographic regions: bird`s-eye views. Kansas Geological Survey, the University of Kansas, Lawrence.

Alexander, G. G., and Allan, J. D. 2007. Ecological success in stream restoration: case studies from the midwestern United States. Environmental Management 40(2):245-255.

Allert, A. L., Fairchild, J. F., Schmitt, C. J., Besser, J. M., Brumbaugh, W. G., and Olson, S. J. 2009. Effects of mining-derived metals on riffle-dwelling benthic fishes in Southeast Missouri, USA. Ecotoxicology and Environmental Safety 72(6):1642-1651.

Allert, A. L., DiStefano, R. J., Fairchild, J. F., Schmitt, C. J., McKee, M. J., Girondo, J. A., Brumbaugh, W. G., and May, T. W. 2013. Effects of historical lead–zinc mining on riffle-dwelling benthic fish and crayfish in the Big River of southeastern Missouri, USA. Ecotoxicology 22(3):506-521.

Angelo, R. T., Cringan, M. S., Chamberlain, D. L., Stahl, A. J., Haslouer, S. G., and Goodrich, C. A. 2007. Residual effects of lead and zinc mining on freshwater mussels in the Spring River Basin (Kansas, Missouri, and Oklahoma, USA). Science of the Total Environment 384:467-496.

Authman, M. M., Zaki, M. S., Khallaf, E. A., and Abbas, H. H. 2015. Use of fish as bio indicator of the effects of heavy metals pollution. Journal of Aquaculture Research & Development 6(4):1-13.

Boroughs, K.L. 2020. Comparison of threatened Neosho madtom densities between riverscapes differing in anthropogenic stressors, with a particular focus on recovery from mining-derived metal pollution. Master`s Thesis, Pittsburg State University, Pittsburg, KS.

Boschung, H. T. and Mayden R. L. 2004. Fishes of Alabama. Smithsonian Institution, Washington.

Brabec, K., Zahrádková, S., Pařil, P., Němejcová, D., Kokeš, J., and Jarkovský, J. 2004. Assessment of organic pollution effect considering differences between lotic and lentic stream habitats. In Integrated assessment of running waters in Europe. Springer, Dordrecht 331-346.

Chambers, D. K., Arruda, J. A., and Jaywardhana A. A. 2005. A synoptic water quality survey of the Spring River and its tributaries. Transactions of the Kansas Academy of Science 108:47-56.

Champagne, C. M. 2011. Diet and Feeding-Related Morphometrics of the Blackstripe Topminnow, *Fundulus notatus*, in Allotropic and Syntopic Populations with the Blackspotted Topminnow, *Fundulus olivaceus*. Masters thesis, University of Southern Mississippi. Hattiesburg, 1-40.

Chen, J. and Chang, H. 2019. Dynamics of wet-season turbidity in relation to precipitation, discharge, and land cover in three urbanizing watersheds, Oregon. River Research and Applications 35(7):892-904.

Collett, D. 2002. Modelling binary data. Second Edition. Chapman and Hall, Boca Raton, Florida, USA.

Davis, J.V. and Schumacher, J.G. 1992. Water-quality characterization of the Spring River Basin, southwestern Missouri and southeastern Kansas (No. 90-4176). US Department of the Interior, US Geological Survey.

Duvernell, D. D., Schaefer, J. F., Hancks, D. C., Fonoti, J. A., and Ravanelli, A. M. 2007. Hybridization and reproductive isolation among syntopic populations of the topminnows *Fundulus notatus* and *F. olivaceus*. Journal of Evolutionary Biology 20(1):152-164.

Duvernell, D. D., and Schaefer, J. F. 2014. Variation in contact zone dynamics between two species of topminnows, *Fundulus notatus* and *F. olivaceus*, across isolated drainage systems. Evolutionary Ecology 28(1):37-53.

Duvernell, D.D., Westhafer, E., and Schaefer, J.F. 2019. Late Pleistocene range expansion of North American topminnows accompanied by admixture and introgression. Journal of Biogeography 46(9):2126-2140.

Edds, D. R. and Dorlac, J. H. 1995. Survey of the fishes of the Spring River basin in Missouri, Kansas, and Oklahoma, with emphasis on the Neosho Madtom. Final report of contract number 710 for the period June 1993-October 1994. Kansas Department of Wildlife and Parks, Pratt, Kansas.

Fausch, K. D., Lyons, J., Karr, J. R., and Angermeier P. L. 1990. Fish communities as indicators of environmental degradation. Pages 123-144 *in* S. M. Adams, editor. Biological indicators of stress in fish. American Fisheries Society, Symposium 8, Bethesda, Maryland.

Fransen, C. M. 2006. Small-bodied fishes of Tar Creek and other small streams in Ottawa

County, Oklahoma. Proceedings of the Oklahoma Academy of Science 9-16.

Grabarkiewicz, J. D., and Davis W. S. 2008. An introduction to freshwater fishes as biological indicators. U.S. Environmental Protection Agency, Office of Environmental Information EPA-260-R-08-016, Washington D.C.

Happel, A. and Gallagher, D. 2022. Decreases in wastewater pollutants increased fish diversity of Chicago's waterways. Science of The Total Environment, 824: 153776.

Hitchman, S.M., Mather, M. E., Smith J. M., and Fencl., J. S. 2018. Identifying keystone habitats with a mosaic approach can improve biodiversity conservation in disturbed ecosystems. Global Change Biology 24:308-321.

Holcroft, N. I. 2004. First record of *Fundulus olivaceus* (Atherinomorpha: Fundulidae) in Kansas. The Southwestern Naturalist 49:87-88.

Hughes, R. M., and Gammon, J. R. 1987. Longitudinal changes in fish assemblages and water quality in the Willamette River, Oregon. Transactions of the American fisheries Society 116:196-209.

Jeffree, R. A., Twining, J. R., and Thomson, J. 2001. Recovery of fish communities in the Finniss River, northern Australia, following remediation of the Rum Jungle uranium/copper mine site. Environmental Science & Rechnology 35: 2932-2941.

Jelks, H. L., S. J. Walsh, N. M. Burkhead, S. Contreras-Balderas, E. Diaz-Pardo, D. A. Hendrickson, J. Lyons, N. E. Mandrak, F. McCormick, J. S. Nelson, S. P. Platania, B. A. Porter, C. B. Renaud, J. J. Schmitter-Soto, E. B. Taylor, and M. L.

Warren Jr. 2008. Conservation status of imperiled North American freshwater and diadromous fishes. Fisheries 33:372-407.

Juracek, K. E. 2006. Sedimentation and occurrence and trends of selected chemical constituents in bottom sediment, Empire Lake, Cherokee County, Kansas, 1905-2005. US Geological Survey.

Kansas Fishes Committee. 2014. Kansas fishes. University Press of Kansas, Lawrence.

Karr, J. R., Fausch, K. D., Angermeier, P. L., Yant, P. R., and Schlosser, I. J. 1986. Assessing biological integrity in running waters. A method and its rationale. Illinois Natural History Survey, Champaign, Special Publication 5:1-28.

Katz, M., and Gaufin, A. R. 1953. The effects of sewage pollution on the fish population of a midwestern stream. Transactions of the American Fisheries Society 82:156-165.

King, A. D., Whitney, J. E., Holloway, J. A., and Boroughs, K. B. 2021a. Lost from Oz? Status of the Sunflower State`s Ozarkian fish fauna. Transactions of the Kansas Academy of Science 124:45-57.

King, A. D., Whitney, J. E., Holloway, J. A., & Boroughs, K. B. 2021b. A black spot on our record: invasion history of the nonnative Blackspotted Topminnow (*Fundulus olivaceus*) in the Spring River subbasin of Kansas, with a comparison to long-term trends in prevalence of Blackstripe Topminnow (*Fundulus notatus*). Transactions of the Kansas Academy of Science 124:216-226.

Mebane, C. A., Eakins, R. J., Fraser, B. G., and Adams, W. J. 2015. Recovery of a mining damaged stream ecosystem. Elementa: science of the Anthropocene 3.

Park, H., Noh, K., Min, J. J., and Rupar, C. 2020. Effects of Toxic Metal Contamination in the Tri State Mining District on the Ecological Community and Human Health: A systematic Review. International Journal of Environmental Research and Public Health 17(18):6783.

Pflieger, W.L. 1997. The Fishes of Missouri. Conservation Commission of the State of Missouri, Jefferson City.

Puckett, L. J. 1995. Identifying the major sources of nutrient water pollution. Environmental Science & Technology, 29:408-414.

R Core Team. 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. [URL https://www.R-project.org/]. Date accessed [January 2022].

Ryon, M. G. 2011. Recovery of fish communities in a warm water stream following pollution abatement. Environmental Management 47:1096-1111.

Schaefer, J. F., Duvernell, D. D., Kreiser, B. R., Champagne, C., Clark, S. R., Gutierrez, M., Stewart, L.K., and Coleman, C. 2012. Evolution of a sexually dimorphic trait in a broadly distributed topminnow (*Fundulus olivaceus*). Ecology and evolution 2:1371-1381.

Schaefer J.F. 2014a. Blackspotted Topminnow, *Fundulus olivaceus.* Storer 1845. pp. 335 336 *in* Kansas Fishes Committee. Kansas Fishes. University of Press of Kansas, Lawrence.

Schaefer J.F. 2014b. Blackstripe Topminnow, *Fundulus notatus.* Rafinesque 1820. pp.

333-334 *in* Kansas Fishes Committee. Kansas Fishes. University of Press of Kansas, Lawrence.

Steffensmeier, N., Remex, N.S., Hrabik, R., and Duvernell, D.D. 2020. Status of the Blackstripe (*Fundulus notatus*) and Blackspotted (*Fundulus olivaceus*) Topminnows in the Ozark uplands of Central Missouri. Southeastern Fishes Council Followings. The University of Tennessee, Knoxville 1(59):14-23.

Thomerson, J. E., and Wooldridge, D. P. 1970. Food habits of allotopic and syntopic populations of the topminnows *Fundulus olivaceus* and *Fundulus notatus*. American Midland Naturalist 84:573-576.

USFWS (US Fish and Wildlife Service). 1991. Neosho Madtom recovery plan. US Fish and Wildlife Service, Denver, Colorado.

Warton, D. I., and Hui, F. K. 2011. The arcsine is asinine: the analysis of proportions in ecology. Ecology 92(1):3-10.

Whitney, J.E., J.A. Holloway, D.T. Scholes, and A.D. King. 2018. Discovery of the Arkansas Darter (*Etheostoma cragini*) in the Cow Creek watershed, Cherokee county, southeastern Kansas. Transactions of the Kansas Academy of Science 121:411-416.

Whitney, J. E., Holloway, J. A., Scholes, D. T. and King, A. D. 2019. Long-term change of fish communities in a polluted watershed: does cleaner water “act” on fishes? Transactions of the American Fisheries Society 148(1):191-206.

Wildhaber, M. L., Allert, A. L., Schmitt, C. J., Tabor, V. M., Mulhern, D., Powell, K. L., and Sowa, S. P. 2000. Natural and anthropogenic influences on the distribution of the threatened Neosho madtom in a Midwestern warmwater stream. Transactions of the American Fisheries Society 129(1):243-261.

Wilkinson, C. and Edds, D. 1996. Biological survey of the Spring River basin in Missouri, Kansas and Oklahoma, with emphasis on the Neosho madtom. Final report of contract 710 for the period June 1994-December 1995. Kansas Department of Wildlife and Parks, Pratt, Kansas.

Wilkinson, C. D., and Edds D. R. 2001. Spatial pattern and environmental correlates of a Midwestern stream fish community: including spatial autocorrelation as a factor in community analyses. American Midland Naturalist 146(2):271-289.

Vuori, K. M. 1995. Direct and indirect effects of iron on river ecosystems. In Annales Zoologici Fennici. Finnish Zoological and Botanical Publishing Board 317-329.

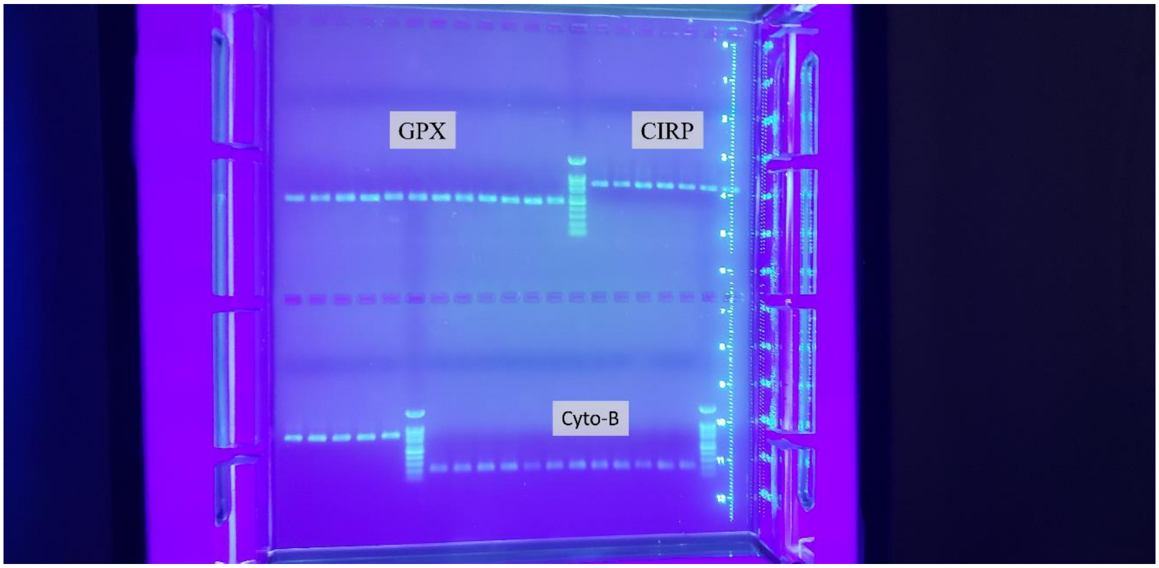
Yount, J. D., and Niemi, G. J. 1990. Recovery of lotic communities and ecosystems from disturbance—a narrative review of case studies. Environmental management 14(5):547-569.

Zeitoun, M. M., and Mehana, E. E. 2014. Impact of water pollution with heavy metals on fish health: overview and updates. Global Veterinaria 12(2):219-231.

APPENDICES

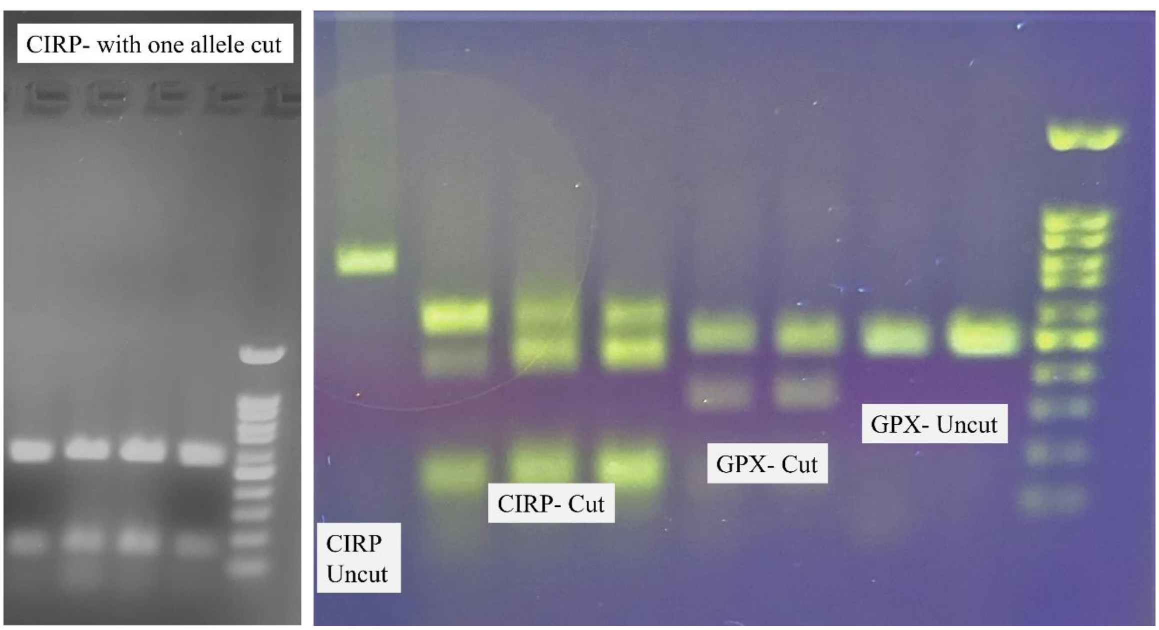
Appendix A

Agarose gel (1%) of PCR primers Cytochrome-B (bottom right), CIRP (bottom left-top right), and GPX (top left) and Bullseye 100BP DNA Ladder for identifiable band reference.



Appendix B

Sample guide to distinguish *Fundulus* species based on their PCR-RFLP banding pattern.



Appendix C

PCR-RFLP assay (1.5% agarose gel) of *Fundulus* individuals collected from the sites above and directly below Empire Lake.