EASTERN PLAINS NATIVE FISH RESEARCH

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The results of the research investigations contained in this report represent work of the authors and may or may not have been implemented as Colorado Parks & Wildlife policy by the Director or the Wildlife Commission.

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COLORADO EASTERN PLAINS NATIVE FISH PROJECT SUMMARY

Period Covered: April 1, 2023 to March 31, 2024

PROJECT OBJECTIVE: To assist in the conservation of native Great Plains fish species in Colorado.

PUBLICATIONS

Fitzpatrick, R. M., D. L. Longie, R. J. Friebertshauser, and H. P. Foutz. 2023. Evaluation of a prefabricated fish passage design for Great Plains fishes. Fishes 8(8), 403. https://doi.org/10.3390/fishes8080403

Kopack, C. J., E. R. Fetherman, D. E. Broder, **R. M. Fitzpatrick**, and L. M. Angeloni. 2023. The effects of environmental enrichment on behavior, morphology, and survival of a species of conservation concern. Conservation Science and Practice. https://doi.org/10.1111/csp2.12999

Swarr, T. R., C. A. Myrick, and **R. M. Fitzpatrick**. *Under review*. Effects of slope on small-bodied fish passage success in an experimental rock ramp fishway. Transactions of the American Fisheries Society.

PRESENTATIONS

Fitzpatrick, R. M., A. J. Piaggio, M. W. Hopken, and R. J. Friebertshauser. Environmental DNA metabarcoding for Great Plains fishes: a comparison to conventional sampling methods. Continuing Education Course for the Colorado/Wyoming Chapter of the American Fisheries Society. Laramie, Wyoming. February 27, 2024.

Fitzpatrick, R. M. Plains fish research projects update. Colorado Parks and Wildlife Aquatic Biologist Meeting. Nathrop, Colorado. February 6, 2024.

Rinker, C. M., C. M. Adams, D. L. Winkelman, **R. M. Fitzpatrick**, and P. A. Schaffer. Intra-Atrial Nematodiasis in Johnny Darters (*Etheostoma nigrum*) in Colorado. American College of Veterinary Pathologists Annual Meeting. Chicago, Illinois. October 31, 2023.

PEER REVIEWS

Ichthyological Research

North American Journal of Fisheries Management

RESEARCH PRIORITY

Incorporating environmental DNA metabarcoding into the plains fish monitoring protocol.

OBJECTIVES

This project will incorporate environmental DNA metabarcoding into CPW's plains sampling protocol to detect threatened and endangered fish, detect aquatic invasive species, and guide future sampling efforts.

COLLABORATORS

- Dr. Toni Piaggio, Research Biologist, National Wildlife Research Center, USDA APHIS, Wildlife Services
- Dr. Matthew Hopken, APHIS Science Fellow, USDA, APHIS, Wildlife Services
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- Ryan Friebertshauser, Research Associate II, Larval Fish Laboratory, Department of Fish, Wildlife, and Conservation Biology, Colorado State University

EXECUTIVE SUMMARY

This section summarizes the findings of a research project funded by Colorado Parks and Wildlife in collaboration with the USDA National Wildlife Research Center investigating the use of environmental DNA (eDNA) metabarcoding in detecting Great Plains fishes. This technique provides the potential for a much less expensive, non-invasive, sampling technique compared to traditional techniques (i.e., electrofishing and seining) that are staff, gear, and time intensive, and risk harm to fishes. Additionally, detecting small-bodied organisms in large river systems can be difficult.

Species monitoring using eDNA is a powerful new technique for wildlife detection that may improve the efficiency of Great Plains fish sampling efforts. Studies comparing eDNA to electrofishing have demonstrated that eDNA is more sensitive for species detection. To improve efficiency and replicability of field sampling, the Smith Root backpack eDNA sampler was designed by a team of molecular ecologists and engineers for high-throughput eDNA sample collection. It is designed to sample larger volumes of water and self-contained sampling capability, which reduces the risk of contamination compared to other eDNA sampling methods. Therefore, this system could easily be incorporated into CPW's existing sampling protocol and provide additional information regarding species distributions, especially for hard to detect species.

At comparative sites, we found eDNA to be similar in species detection to electrofishing. In some cases, the eDNA approach detected a broader diversity of fish species. Across all sites, the mean number of species detected with eDNA was 11.5 (range: 8–19) while electrofishing detected 9.7 (range: 5–20) (Tables 4–17). The mean number of unique detections at a site (*i.e.*, detected by one method but not the other) for eDNA was 3.5 (range: 0–10) and for electrofishing it was 1.7 (range: 0–6). There were a few instances of contamination with the Smith Root eDNA sampler. These issues were tested against laboratory procedures, and it was determined that the contamination was occurring in the field. Although the contamination issue needs resolution, we still had the ability to detect fish species diversity associated with the sampled watershed that corresponded to electrofishing and historical records. Future work will replicate the comparative site analysis, but with additional and improved decontamination procedures occurring between sampling sites instead of only between major watersheds. Although there will always be a need for traditional sampling, this study demonstrates that eDNA is another useful tool for Colorado Great Plains fish species detection that is non-invasive and provides valuable data about species diversity and presence/absence.

INTRODUCTION

Fed by the eastern slope of the Rocky Mountains in its headwaters and maintained by groundwater and precipitation in lower reaches (Fausch and Bramblett 1991), rivers and streams in the western Great Plains offer a broad diversity of geomorphology, hydrology, and ultimately habitat for aquatic taxa (Fausch and Bestgen 1997). This diversity in habitat, and thereby fish communities, is largely apparent in the eastern Great Plains of Colorado where assemblage shifts can be seen longitudinally from the Rocky mountain headwaters to the eastern border and the mountain-plains transition zone occurring between them (Rahel and Hubert 1991; Haworth et al. 2020). Despite this unique zonation of fish assemblages, species richness in this ecoregion is less than that of more mesic drainages (Fausch and Bestgen 1997) likely due to relatively simple habitat, harsh physiochemical attributes (Matthews 1987), and historical, intermittent flow during the dry season (Magoulick and Kobza 2003). While adaptations to this dynamic environment may suggest resiliency, contemporary, anthropogenic disturbances such as groundwater pumping (Falke et al. 2011), introduction of nonnative taxa, and stream fragmentation due to the installation of instream structures (Walters et al. 2014; Richer et al. 2020) greatly imperil taxa native to this ecoregion. Among the 30 extant species native to the South Platte, Arkansas, and Republican River drainages in Colorado, 13 are currently listed as threatened or endangered by Colorado Parks and Wildlife (Colorado Parks and Wildlife, 2022).

An alternative method to conventional sampling techniques in lotic systems has emerged through the ability to accurately collect and assign taxonomy to extra-organismal DNA (eDNA) (Taberlet et al. 2018). Through successful collection and amplification of genetic material, emanating from shed scales, slime, feces, etc. (Jerde et al. 2011; Rees et al. 2014), researchers and managers alike have expanded distribution knowledge (Schmelzle and Kinziger 2016; Janosik and Johnston 2015), improved the ability of early detection of

invasives (Goldberg et al. 2013; Xia et al. 2018; Whitaker et al. 2021), and increased detection ability for rare or elusive species (Johnston and Janosik 2019) across a wide range of aquatic taxa. Multiple comparative studies have observed eDNA performing equally as well or better than conventional, aquatic sampling methods in terms of detection (Evans et al. 2016; Shaw et al. 2016; Valentini et al. 2016; McColl-Gausden et al. 2021). Further, sampling through eDNA not only permits sites to be surveyed that are normally inacessible by traditional methods (McColl-Gausden et al. 2021) but requires less time, effort, and equipment as well (Pfleger et al. 2016). While eDNA studies have historically focused on single-species detection (McColl-Gausden et al. 2021), an emerging technique, termed metabarcoding, expands upon the method through it's ability to produce species-richness data (Tsuji et al. 2019); a metric paramount to freshwater conservation (Su et al. 2021).

Through the use of high-throughput sequencing and clade-, as opposed to species-specific primer sets, metabarcoding can reveal species compositions from single collection event (Deagle et al. 2014; Miya et al. 2015; Deiner et al. 2017; Yamamoto et al. 2017). Rapid biodiversity assessments of this nature can be designed to not only identify native communities but invasive taxa as well which can be crucial in the early detection and management of previously unknown invaders (Brown et al. 2016; Borrell et al. 2017). While the preparation and laboratory processes associated with metabarcoding are far more in depth than single-species eDNA work (McColl-Gausden et al. 2020), the information produced ultimately leads towards a less time consuming, and potentially more sensitive survey method thereby reducing the strains of empirical sampling listed above and ultimately expanding the reach of biologists and managers working in eastern plains systems.

To validate the use of eDNA as a complimentary survey method, we are conducting a comparative study investigating the efficacy of eDNA and conventional methods at paired sites across the eastern plains of Colorado. While most comparative studies of this nature have taken a single-species approach across temporally disparate sampling events (McColl-Gausden et al. 2020), the current work remedies this by taking temporally paired metabarcoding samples. We hypothesized that eDNA-metabarcoding samples will perform equally as well or better than conventional sampling in regards to detection probability (sensitivity) and measurements of alpha diversity. We additionally developed a sampling protocol for the field designed to be accessible, repeatable, and accurate regardless of a collector's background in molecular ecology (Friebertshauser et al. 2020). Our primary aim was to develop and validate an alternative and complimentary survey technique that will assist in the limited effort conservation biologists and managers have to monitor and conserve fishes native to the eastern plains ecoregion of Colorado.

METHODS

Primer Selection and Local Reference Database Development

Taxonomic identification of multiple species from eDNA relies on the ability to compare unidentified reads from next-generation sequencing efforts to a reference database of known sequences (Taberlet et al. 2018). Therefore, not only the completeness of a

reference database but the primer(s) chosen when metabarcoding will have an impact on the accuracy and coverage of a protocol. Due to its variation among species and taxonomically expansive reference library (iBOL, International Barcode of Life Consortium 2016), the mitochondrial cytochrome oxidase subunit I (COI) has become widely used in metabarcoding studies (Deagle et al. 2014). However, certain drawbacks apparent in the COI subunit (Deagle et al. 2014) have lead many studies focused on metabarcoding of fish communities towards amplifying fragments from the 12s and 16s regions of mitochondrial rRNA (Miya et al. 2015; Evans et al. 2016; Lecaudey et al. 2019). Additionally, the use of multiple primers has been observed to increase the taxonomic breadth of detection (Evans et al. 2016; Schenekar et al. 2020), therefore, the current study used both a 16s (16S fish-specific, Shaw et al. 2016) and 12s (MiFish-U, Miya et al. 2015) region of mitochondrial rRNA. These primers were designed to amplify fragments of ~100 bp (base pairs) and 163-185 bp respectively. These relatively short amplicon lengths are ideal for eDNA application as longer fragments will degrade more quickly in an extraorganismal environment than shorter ones (Taberlet et al. 2018). Since databases for these regions are relatively less complete than those for COI regions (Weigand et al. 2019), we compiled a local reference database containing 12s and 16s sequences from species of interest within the eastern plains of Colorado.

Up to five fin clips per target species were collected across the South Platte and Arkansas River basins. Target species fell into one of three distribution statuses: Native, Invasive, or Potentially Invasive. Fin clips were collected using sterilized dissection scissors and stored in 100% molecular grade ethanol (EtOH) at room temperature until extraction. Tissues were extracted using a DNeasy Blood and Tissue Kit in conjunction with a QIAcube Automated DNA Isolation and Purification system (QIAGEN, Hilden, Germany). Prior to purification via the QIAcube, tissues were digested in a lysis solution of 180 µl Buffer ATL and 20 µl proteinase K for 24 hr on a dry bath at 56°C.

PCR for the 16S1 primer was carried out using a 20 µl reaction containing 10.3 µl molecular grade H_2O, 4 µl 5x PCR buffer (Promega Corporation, Madison, Wisconsin, United States), 0.2 µl of $MgCl_2$ (25mM), 2 µl dNTPs (10mM), 1 µl forward and reverse primer (10 µM), 0.5 µl GoTaq polymerase (5 U/µl) (Promega Corporation, Madison, Wisconsin, United States), and 1 µl template. The 35 cycle thermal cycling profile after an initial 4 min denaturation at 95°C was as follows: denaturation at 95°C for 15 s; annealing at 55°C for 30 s; and extension at 72°C for 30 s. A final extension step occurred at 72°C for 5 min.

PCR for the MiFish-U primer was carried out using a 15 µl reaction containing 7.6 µl molecular grade H_2O, 3 µl 5x PCR buffer, 0.9 µl of $MgCl_2$ (25mM), 0.5 µl dNTPs (10mM), 0.75 µl forward and reverse primer (10 µM), 0.5 µl GoTaq polymerase (5 U/µl), and 1 µl template. The 30 cycle thermal cycling profile after an initial 2 min denaturation at 94°C was as follows: denaturation at 98°C for 5 s; annealing at 50°C for 10 s; and extension at 72°C for 10 s. A final extension step occurred at 72°C for 5 min.

Prior to cycle sequencing, unincorporated primers and dNTP's were removed from PCR products with Exo SAP-IT (Applied Biosystems, Waltham, Massachusetts, United

States). Cycle sequencing was conducted in both directions using BigDye Terminator following the manufacturer's protocol (v3.1, Applied Biosystems, Waltham, Massachusetts, United States) and run in a 10 µl reaction: 5.475 µl H_2O, 2.275 µl 5x sequencing buffer, 0.25 µl BigDye Terminator, 1 µl primer, and 1 µl template. The 35 cycle thermal cycling profile after an initial 1 min denaturation at 96°C was as follows: denaturation at 96°C for 10 s; annealing at 50°C for 30 s; and extension at 60°C for 4 min. To remove unincorporated dye-terminators, products were then passed through a UNIFILTER microplate (Cytiva, Marlborough, Massachussetts, United States) filled with a Sephadex (Cytiva, Marlborough, Massachussetts, United States) preparation. Purified products from both forward and reverse strands were then Sanger sequenced on a 3500xL genetic analyzer (Applied Biosystems, Waltham, Massachussetts). Sequences were edited and aligned using Sequencher (version 5.4.6, Gene Codes Corporation) and uploaded to a custom, relational database. Species identification was verified against reference sequences in GenBank using the Basic Local Alignment Search Tool (BLAST, Altschul et al. 1990). In total, 361 sequences (both 12s and 16s) were uploaded to the reference database comprising 8 orders, 13 families, and 39 species (Sequenced taxa, S3).

Sampling Sites

Comparative sampling sites were chosen based on conventional fish community sampling conducted by Colorado Parks and Wildlife during the fall of 2021. All sampling sites (n=13) occurred east of the continental divide in Colorado and within the South Platte and Arkansas River basins. Comparative sites were separated by >/= 5 river km in order to increase the probability of detecting novel, genetic material in eDNA samples (Wilcox et al. 2016; Wacker et al. 2019; Bedwell and Goldberg 2020). Sampling sites in the South Platte River basin (n=9) took place across two of the three major physiographic regions. These regions consist of montane streams, eastern plains streams and the transition zone that divides the two. Montane and eastern plains streams are largely differentiated by gradient, channel morphology, temperature (Fausch and Bestgen 1997), and species assemblage (Rahel and Hubert 1991) while the transition zone describes an ecotone between them (Propst 1982) thereby supporting unique fish assemblages (Bestgen et al. 2017; Haworth et al. 2020). Three sites were sampled within the transition zone of the South Platte River basin: West Plum Creek (WP1, WP2, and WP3, Douglas County), East Plum Creek (EP1, Douglas County), St. Vrain Creek (SV1, Boulder County), and Left Hand Creek (LH1, Boulder County). Sample reaches in the transition zone were characterized by narrow channels, cobble-gravel substrate, and relatively cooler water. The remaining three sites sampled in this drainage occurred in the eastern plains physiographic region: Lodgepole Creek (LP1, Sedgwick County), South Platte River (SP1, Morgan County and SP2, Logan County). Except for the site on Lodgepole Creek (a tributary to the South Platte River with a relatively narrow channel), reaches in this physiographic region are defined by wide, braided channels, sandy substrate, and low gradient. Four sites were sampled along Fountain Creek (FC1, FC2, and FC3, El Paso County; FC4, Pueblo County); a tributary to the Arkansas River. These sites occur at the eastern terminus of the transition zone and accordingly resemble eastern plains streams in their hydrology and geomorphology. Fountain Creek sites historically contain a reduced

species assemblage compared to reaches within the South Platte River basin (Colorado Parks and Wildlife, 2021).

Environmental DNA Sampling and Filter Extraction

Environmental DNA samples were collected at comparative sites on the same day, just prior to conventional fish sampling or any disturbance of the sampling reach (Figure 1). Sampling occurred during September and October of 2021. While a variety of methods for the collection of aquatic eDNA exist (Tsuji et al. 2019), samples were filtered *in situ* using the Smith-Root eDNA Sampler (Smith-Root, Inc., Vancouver, Washington, United States) (Figure 1). This unit not only allows for on-site filtration, which has been shown to increase detection, (Yamanaka et al. 2016) but decreases sampling time and limits contamination potential through its design and single-use filter housings (Thomas et al. 2018). Through the use of the Smith-Root telescopic sampling pole and trident head attachment, 3, 2, and l samples were able to be collected simultaneously (Figure 1). Filtration parameters of the unit were based on a comparative study (Thomas et al. 2018): flow rate of 1 l/min, maximum pressure of 10 PSI, and use of a 5µm, 47 mm diameter filter. Immediately following on-site filtration, filter discs were placed in a 2 ml cryovial filled with Longmire's buffer (Longmire et al. 1997) and stored at 4°C until extraction. One field negative per site was filtered and stored in the same manner as above using distilled water. Filtration was conducted at the downstream-most point of each paired, traditional sampling reach. Decontamination was done following manufacturer's protocols when sampling between main watersheds (South Platte and Arkansas River basins).

Extraction methods were modified from Spens et al. (2017) and Miya et al. (2015) and are provided in detail in Friebertshauser (2022). The filter was first cut in half with each half being placed into an individual 2 ml safe-lock microcentrifuge tube. Remaining Longmire's buffer (~1 ml) was then transferred equally among two, 2 ml safe-lock microcentrifuge tubes. Half of the filter disc and volume of Longmire's buffer was archived prior to extraction. Unarchived Longmire's buffer was then centrifuged at 6,000 x g for 45 min in order to pelletize genetic material. After removing the supernatant, each pellet and filter half was submerged in 100 µl and 300 µl of lysis working solution (90% ATL buffer, 10% Proteinase K) respectively. Filters and pellets were digested overnight in independent vessels on a shaking dry bath at 56°C rotating at 80 rpm. The following day, contents from the digested filters were transferred to a modified mini spin column (QIAGEN, Hilden, Germany) with the filter membrane removed. Spin-columns were then centrifuged at 6,000 x g for 5 min into the collection tubes containing the digested pellet in order to concatenate DNA from both sources. Half of the digested solution (200 µl) was purified with the Dneasy Blood and Tissue kit in conjuction with the QIAcube Automated DNA Isolation and Purification system. The remaining volume was archived.

Conventional Fish Sampling

Conventional fish sampling was conducted as part of Colorado Parks and Wildlife's annual stream monitoring and occurred immediately following filtration of eDNA samples. Sites within the South Platte River basin were sampled using a three-pass removal technique where the first two passes consisted of electrofishing with either a

Smith-Root VVP-15B electrofishing barge or three, LR-24 electrofishing backpacks depending on the water depth at each site. The third pass consisted of multiple seining efforts with a 4.7-mm mesh size seine. Fishes were held in live wells between passes. Sampling within the Arkansas River drainage consisted of single-pass electrofishing with two, Smith-Root LR-24 electrofishing. All fishes collected during traditional sampling were enumerated and identified to species. Sampling reaches ranged in length from 81.7 to 211.8 m ($\bar{x}$ = 148.9 m). Habitat measurements were additionally collected during each sampling event (e.g. pH, turbidity, water temperature, and measurements of channel morphology).

Library Preparation and Sequencing

Metabarcoding libraries were prepared using a two-step PCR strategy similar to that used by Hopken et al. (2021). The first round PCR targeted fragments using the 16s fish-specific MiFish-U primers (Table 1). Both primers were modified to include heterogeneity spacers, in order to improve sequencing quality, and Illumina sequencing primers were added at the 5' end in order to add indexes and Illumina sequencing adaptors in the second PCR (Illumina, Sand Diego, CA, United States). Primers were unable to be multiplexed due to divergent annealing temperatures. PCR using the 16s fish-specific primers was carried out using a 25 µl reaction containing 3.5 µl molecular grade H_2O, 12.5 µl 2x QIAGEN Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 2.5 µl of 2 µM forward and reverse primer, and 4 µl template. The 40-cycle thermal cycling profile after an initial 15 min denaturation at 95°C was as follows: denaturation at 94°C for 30 s; annealing at 52°C for 90 s; and extension at 72°C for 120 s. A final extension step occurred at 72°C for 10 min.

Table 1. Primers for database development and first round PCR.

Primer Name	Sequencing Primer	Heterogeneity Spacer	Region of Interest
16S1 F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	NNNNNN	GTCGGTAAAACTCGTGCCAGC
16S1 R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	NNNNNN	CATAGTGGGGTATCTAATCCCAGTTTG
MiFish-U F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	NNNNNN	GGTCGCCCCAACCRAAG
MiFish-U R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	NNNNNN	CGAGAAGACCCTWTGGAGCTTIAG

PCR using the MiFish-U primers was carried out using a 25 µl reaction containing 3.5 µl molecular grade H_2O, 12.5 µl 2x QIAGEN Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 2.5 µl of 2 µM forward and reverse primer, and 4 µl template. A touchdown PCR method, modified from Gold et al. (2021), was employed: initial 15 min denaturation at 95°C followed by 13 cycles of denaturation at 94°C for 30 s, annealing for 39 s beginning at 69.5°C and decreasing by 1.5°C every cycle, and extension at 72°C for 60 s. Thirty additional cycles were carried out with an annealing temperature at 50°C followed by a final extension at 72°C for 10 min.

Dual-indices and Illumina sequencing adaptors were added to first-round PCR products (Table 2) through a 15 µl reaction containing 2.9 µl molecular grade H_2O, 7.5 µl 2x QIAGEN Multiplex PCR Master Mix, 1.8 µl of forward and reverse, indexed primers, and 1 µl of undiluted product from the first-round PCR. The 8 cycle thermal cycling profile after an initial 15 min denaturation at 95°C was as follows: denaturation at 95°C for 15 s; annealing at 55°C for 45 s; and extension at 72°C for 60 s. A final extension step occurred at 72°C for 10 min.

Table 2. Primers for second round of PCR.

Primer name	Flowcell Adaptor	I5/I7 Indexes	Sequencing primer
PCR2-P5	AATGATACGGCGACCACCGAGATCTACAC	XXXXXXXX	TCGTCGGCAGCGTC
PCR2-P7	CAAGCAGAAGACGGCATACGAGAT	XXXXXXXX	GTCTCGTGGGCTCGG

Following each PCR step, products were checked for successful amplification using a QIAxcel fragment analyzer (QIAGEN, Hilden, Germany) and cleaned (removal of unincorporated primers, DNTP's, and primer dimers) using 1.4x Mag-Bind TotalPure NGS magnetic beads following the manufacturer's protocol (Omega Bio-Tek Inc., Norcross, Georgia, United States). Concentrations of second-round PCR products were calculated using a Qubit dsDNA HS Assay Kit (Invitrogen, Carslbad, CA, United States) and then pooled in equimolar volumes. Final library quantification was conducted using a KAPA Library Quantification Kit (Roche Holding AG, Basel, Switzerland). The 20 µl reaction volume consisted of 12 µl KAPA SYBR FAST qPCR master mix/primer premix, 4 µl molecular grade H_2O, and 4 µl of the library diluted to both 1:1000 and 1:10000. The 35 cycle thermal cycling profile after an initial 5 min denaturation at 95°C was as follows: denaturation at 95°C for 30 s and annealing at 60°C for 45 s. Libraries were then run on an Illumina MiSeq System using the cycle MiSeq Reagent Kit v2 (Illumina, San Diego, CA, United States).

Figure 1. Smith Root eDNA backpack sampler. Note the triplicate collection system. Water is collected and run through filters in the blue backpack seen at the feet of the technician. Photo credit: Boyd Wright.

RESULTS

Primer Selection and Local Reference Database Development

In total, 361 sequences (both 12s and 16s) were uploaded to the reference database comprising 8 orders, 13 families, and 39 species (Sequenced taxa, S3).

Environmental DNA Sampling

Sampling resulted in comparative results at 13 sites (Table 3). Across all of the sites, the mean number of species detected with eDNA was 11.5 (range: 8 – 19) while electrofishing detected 9.7 (range: 5 – 20) (Tables 4 -16). The mean number of unique detections at a site (*i.e.*, detected by one method but not the other) for eDNA was 3.5 (range: 0 – 10) and for electrofishing it was 1.7 (range: 0 – 6) (Table 17). At some of the sites, there was contamination in some of the field blanks. None of the laboratory blanks were contaminated, which suggests the contamination happened in the field. There was only one occurrence of cross river basin, *Platygobio gracilis* at EP1, but all other contamination events were species that were detected at that site using electrofishing.

Sampling resulted in comparative results at 13 sites (Table 5). Note: there was an additional eDNA collection made on Fountain Creek, but those results were not able to

be included in this report due to a lack of effort reported. Comparative site results are provided in Tables 6–18.

Table 3. Thirteen sampling efforts to compare eDNA and conventional fish sampling methods.

siteID	Stream	cpwStationCode	sampleDate	lat	lon
SV1	St. Vrain Creek	SP1423	9/21/2021	40.168625	-105.143113
LH1	Lefthand Creek	SP1459	9/23/2021	40.15292	-105.090091
LP1	Lodgepole Creek	SP6726	9/29/2021	40.959648	-102.384566
SP2	South Platte River	SP5908	9/30/2021	40.67764	-103.133536
FC1	Fountain Creek	AR0550	10/5/2021	38.815925	-104.821484
FC2	Fountain Creek	AR3710	10/6/2021	38.732734	-104.733998
FC3	Fountain Creek	AR1834	10/7/2021	38.606522	-104.673077
FC4	Fountain Creek	AR0147	10/7/2021	38.439793	-104.593632
WP1	West Plum Creek	SP7192	10/13/2021	39.293629	-104.974395
WP3	West Plum Creek	SP0012	10/13/2021	39.351488	-104.975569
EP1	East Plum Creek	SP8129	10/14/2021	39.427804	-104.923305
WP2	West Plum Creek	SP7193	10/14/2021	39.370803	-104.962514
SP1	South Platte River	SP7854	10/20/2021	40.325861	-103.578642

Table 4. Comparison of species detection by eDNA and by conventional methods at St. Vrain Creek on St. Vrain Creek on 9/21/2021.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
SV1	St. Vrain Creek	Creek Chub S*emotilus atromaculatus*	X	X
SV1	St. Vrain Creek	White Sucker *Catostomus commersonii*	X	X
SV1	St. Vrain Creek	Common Shinter *Luxilus cornutus*	X	X
SV1	St. Vrain Creek	Central Stoneroller *Campostoma anomalum*	X	X
SV1	St. Vrain Creek	Longnose Dace *Rhinichthys cataractae*	X	X
SV1	St. Vrain Creek	Brown Trout *Salmo trutta*	X	X
SV1	St. Vrain Creek	Green Sunfish *Lepomis cyanellus*	X	X
SV1	St. Vrain Creek	Largemouth Bass *Micropterus salmoides*	X	X
SV1	St. Vrain Creek	Bluegill *Lepomis macrochirus*	X	X
SV1	St. Vrain Creek	Longnose Sucker *Catostomus catostomus*		X
SV1	St. Vrain Creek	Plains Topmionnow *Fundulus sciadicus*		X
SV1	St. Vrain Creek	Johnny Darter *Etheostoma nigrum*		X

Table 5. Comparison of species detection by eDNA and by conventional methods at Lefthand Creek on 9/23/2021.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
LH1	Lefthand Creek	Creek Chub S*emotilus atromaculatus*	X	X
LH1	Lefthand Creek	Red Shiner *Cyprinella lutrensis*	X	X
LH1	Lefthand Creek	White Sucker *Catostomus commersonii*	X	X
LH1	Lefthand Creek	Longnose Sucker *Catostomus catostomus*	X	X
LH1	Lefthand Creek	Longnose Dace *Rhinichthys cataractae*	X	X
LH1	Lefthand Creek	Green Sunfish *Lepomis cyanellus*	X	X
LH1	Lefthand Creek	Brown Trout *Salmo trutta*	X	X
LH1	Lefthand Creek	Largemouth Bass *Micropterus salmoides*	X	X
LH1	Lefthand Creek	Fathead Minnow *Pimephales promelas*	X	X
LH1	Lefthand Creek	Campostoma_anomalum	X	X
LH1	Lefthand Creek	Western Mosquitofish *Gambusia affinis*		X
LH1	Lefthand Creek	Iowa Darter *Etheostoma exile*		X
LH1	Lefthand Creek	Bluegill *Lepomis macrochirus*		X
LH1	Lefthand Creek	Common Carp *Cyprinus carpio*		X
LH1	Lefthand Creek	Stonecat *Noturus flavus*		X
LH1	Lefthand Creek	Johnny Darter *Etheostoma nigrum*		X
LH1	Lefthand Creek	Flathead Chub *Platygobio gracilis*	X	
LH1	Lefthand Creek	Brook Stickleback *Culaea inconstans*	X	

Table 6. Comparison of species detection by eDNA and by conventional methods at Lodgepole Creek on 9/29/2023.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
LP1	Lodgepole Creek	Red Shiner *Cyprinella lutrensis*	X	X
LP1	Lodgepole Creek	Creek Chub S*emotilus atromaculatus*	X	X
LP1	Lodgepole Creek	Fathead Minnow *Pimephales promelas*	X	X
LP1	Lodgepole Creek	Common Carp *Cyprinus carpio*	X	X
LP1	Lodgepole Creek	Central Stoneroller *Campostoma anomalum*	X	X
LP1	Lodgepole Creek	Sand Shiner *Notropis stramineus*	X	X
LP1	Lodgepole Creek	Suckermouth Minnow *Phenacobius mirabilis*	X	X
LP1	Lodgepole Creek	White Sucker *Catostomus commersonii*	X	X
LP1	Lodgepole Creek	Channel Catfish *Ictalurus punctatus*	X	X
LP1	Lodgepole Creek	Bluegill *Lepomis macrochirus*	X	X
LP1	Lodgepole Creek	Freshwater Drum *Aplodinotus grunniens*	X	X
LP1	Lodgepole Creek	Western Mosquitofish *Gambusia affinis*	X	X
LP1	Lodgepole Creek	Northern Plains Killifish *Fundulus kansae*	X	X
LP1	Lodgepole Creek	Quillback *Carpiodes cyprinus*	X	X
LP1	Lodgepole Creek	Largemouth Bass *Micropterus salmoides*	X	X
LP1	Lodgepole Creek	Green Sunfish *Lepomis cyanellus*		X
LP1	Lodgepole Creek	Brook Stickleback *Culaea inconstans*	X	
LP1	Lodgepole Creek	Gizzard Shad *Dorosoma cepedianum*	X	
LP1	Lodgepole Creek	Bigmouth Shiner *Notropis dorsalis*	X	
LP1	Lodgepole Creek	Smallmouth Bass *Micropterus dolomieu*	X	
LP1	Lodgepole Creek	Walleye *Sander vitreus*	X	
LP1	Lodgepole Creek	Yellow Bullhead *Ameiurus natalis*	X	
LP1	Lodgepole Creek	Black Bullhead *Ameiurus melas*	X	
LP1	Lodgepole Creek	Yellow Perch *Perca flavescens*	X	
LP1	Lodgepole Creek	White crappie *Pomoxis annularis*	X	
LP1	Lodgepole Creek	Black Crappie *Pomoxis nigromaculatus*	X	

Table 7. Comparison of species detection by eDNA and by conventional methods the South Platte River on 10/20/2021.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
SP1	South Platte River	Northern Plains Killifish *Fundulus kansae*	X	X
SP1	South Platte River	Sand Shiner *Notropis stramineus*	X	X
SP1	South Platte River	White Sucker *Catostomus commersonii*	X	X
SP1	South Platte River	Red Shiner *Cyprinella lutrensis*	X	X
SP1	South Platte River	Creek Chub S*emotilus atromaculatus*	X	X
SP1	South Platte River	Fathead Minnow *Pimephales promelas*	X	X
SP1	South Platte River	Bigmouth Shiner *Notropis dorsalis*	X	X
SP1	South Platte River	Freshwater Drum *Aplodinotus grunniens*	X	X
SP1	South Platte River	Western Mosquitofish *Gambusia affinis*	X	X
SP1	South Platte River	Central Stoneroller *Campostoma anomalum*	X	X
SP1	South Platte River	Iowa Darter *Etheostoma exile*		X
SP1	South Platte River	Common Carp *Cyprinus carpio*	X	X
SP1	South Platte River	Johnny Darter *Etheostoma nigrum*		X
SP1	South Platte River	Yellow Bullhead *Ameiurus natalis*	X	
SP1	South Platte River	Brook Stickleback *Culaea inconstans*	X	
SP1	South Platte River	Gizzard Shad *Dorosoma cepedianum*	X	
SP1	South Platte River	Largemouth Bass *Micropterus salmoides*	X	
SP1	South Platte River	Longnose Sucker *Catostomus catostomus*	X	
SP1	South Platte River	Black Bullhead *Ameiurus melas*	X	
SP1	South Platte River	Longnose Dace *Rhinichthys cataractae*	X	

Table 8. Comparison of species detection by eDNA and by conventional methods at the South Platte River on 9/30/2021.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
SP2	South Platte River	Sand Shiner *Notropis stramineus*	X	X
SP2	South Platte River	Bigmouth Shiner *Notropis dorsalis*	X	X
SP2	South Platte River	Brassy Minnow *Hybognathus hankinsoni*	X	X
SP2	South Platte River	White Sucker *Catostomus commersonii*	X	X
SP2	South Platte River	Fathead Minnow *Pimephales promelas*	X	X
SP2	South Platte River	Northern Plains Killifish *Fundulus kansae*	X	X
SP2	South Platte River	Creek Chub S*emotilus atromaculatus*	X	X
SP2	South Platte River	Red Shiner *Cyprinella lutrensis*	X	X
SP2	South Platte River	Central Stoneroller *Campostoma anomalum*	X	X
SP2	South Platte River	Suckermouth Minnow *Phenacobius mirabilis*	X	X
SP2	South Platte River	Common Carp *Cyprinus carpio*	X	X
SP2	South Platte River	Western Mosquitofish *Gambusia affinis*	X	X
SP2	South Platte River	Green Sunfish *Lepomis cyanellus*	X	X
SP2	South Platte River	Longnose Dace *Rhinichthys cataractae*	X	X
SP2	South Platte River	Brook Stickleback *Culaea inconstans*	X	X
SP2	South Platte River	Iowa Darter *Etheostoma exile*		X
SP2	South Platte River	Johnny Darter *Etheostoma nigrum*		X
SP2	South Platte River	Largemouth Bass *Micropterus salmoides*		X
SP2	South Platte River	Orangespotted Sunfish *Lepomis humilis*		X
SP2	South Platte River	Hybrid sunfish		X
SP2	South Platte River	Yellow Bullhead *Ameiurus natalis*	X	
SP2	South Platte River	Gizzard Shad *Dorosoma cepedianum*	X	
SP2	South Platte River	Black Bullhead *Ameiurus melas*	X	
SP2	South Platte River	Channel Catfish *Ictalurus punctatus*	X	

Table 9. Comparison of species detection by eDNA and by conventional methods at Fountain Creek on 10/5/2021.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
FC1	Fountain Creek	White Sucker *Catostomus commersonii*	X	X
FC1	Fountain Creek	Brown Trout *Salmo trutta*	X	X
FC1	Fountain Creek	Longnose Dace *Rhinichthys cataractae*	X	X
FC1	Fountain Creek	Fathead Minnow *Pimephales promelas*		X
FC1	Fountain Creek	Creek Chub S*emotilus atromaculatus*	X	X
FC1	Fountain Creek	Longnose Sucker *Catostomus catostomus*	X	
FC1	Fountain Creek	Common Carp *Cyprinus carpio*	X	
FC1	Fountain Creek	White crappie *Pomoxis annularis*	X	
FC1	Fountain Creek	Northern Plains Killifish *Fundulus kansae*	X	
FC1	Fountain Creek	Cutthroat Trout *Oncorhynchus clarkii*	X	

Table 10. Comparison of species detection by eDNA and by conventional methods at Fountain Creek on 10/6/2021.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
FC2	Founttain Creek	Longnose Dace *Rhinichthys cataractae*	X	X
FC2	Founttain Creek	Flathead Chub *Platygobio gracilis*	X	X
FC2	Founttain Creek	White Sucker *Catostomus commersonii*	X	X
FC2	Founttain Creek	Central Stoneroller *Campostoma anomalum*	X	X
FC2	Founttain Creek	Fathead Minnow *Pimephales promelas*	X	X
FC2	Founttain Creek	Longnose Sucker *Catostomus catostomus*	X	X
FC2	Founttain Creek	Creek Chub S*emotilus atromaculatus*	X	X
FC2	Founttain Creek	Brown Trout *Salmo trutta*	X	
FC2	Founttain Creek	Common Carp *Cyprinus carpio*	X	

Table 11. Comparison of species detection by eDNA and by conventional methods at Fountain Creek on 10/7/2021.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
FC3	Fountain Creek	Flathead Chub *Platygobio gracilis*	X	X
FC3	Fountain Creek	White Sucker *Catostomus commersonii*	X	X
FC3	Fountain Creek	Longnose Dace *Rhinichthys cataractae*	X	X
FC3	Fountain Creek	Central Stoneroller *Campostoma anomalum*	X	X
FC3	Fountain Creek	Longnose Sucker *Catostomus catostomus*	X	X
FC3	Fountain Creek	Common Carp *Cyprinus carpio*	X	
FC3	Fountain Creek	Bigmouth Shiner *Notropis dorsalis*	X	
FC3	Fountain Creek	Fathead Minnow *Pimephales promelas*	X	
FC3	Fountain Creek	Creek Chub S*emotilus atromaculatus*	X	
FC3	Fountain Creek	Brook Stickleback *Culaea inconstans*	X	
FC3	Fountain Creek	Arkansas Darter *Etheostoma_cragini*	X	

Table 12. Comparison of species detection by eDNA and by conventional methods at Fountain Creek on 10/7/2021.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
FC4	Fountain Creek	Flathead Chub *Platygobio gracilis*	X	X
FC4	Fountain Creek	Central Stoneroller *Campostoma anomalum*	X	X
FC4	Fountain Creek	White Sucker *Catostomus commersonii*	X	X
FC4	Fountain Creek	Sand Shiner *Notropis stramineus*	X	X
FC4	Fountain Creek	Longnose Dace *Rhinichthys cataractae*	X	X
FC4	Fountain Creek	Longnose Sucker *Catostomus catostomus*	X	X
FC4	Fountain Creek	Fathead Minnow *Pimephales promelas*	X	X
FC4	Fountain Creek	Northern Plains Killifish *Fundulus kansae*	X	X
FC4	Fountain Creek	Western Mosquitofish *Gambusia affinis*		X
FC4	Fountain Creek	Brook Stickleback *Culaea inconstans*	X	
FC4	Fountain Creek	Arkansas Darter *Etheostoma_cragini*	X	
FC4	Fountain Creek	Common Carp *Cyprinus carpio*	X	
FC4	Fountain Creek	Yellow Bullhead *Ameiurus natalis*	X	

Table 13. Comparison of species detection by eDNA and by conventional methods at West Plum Creek on 10/13/2021.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
WP1	West Plum Creek	Creek Chub S*emotilus atromaculatus*	X	X
WP1	West Plum Creek	Longnose Dace *Rhinichthys cataractae*	X	X
WP1	West Plum Creek	Central Stoneroller *Campostoma anomalum*	X	X
WP1	West Plum Creek	Common Shiner *Luxilus cornutus*	X	X
WP1	West Plum Creek	Johnny Darter *Etheostoma nigrum*	X	X
WP1	West Plum Creek	Brook Stickleback *Culaea inconstans*	X	X
WP1	West Plum Creek	White Sucker *Catostomus commersonii*	X	X
WP1	West Plum Creek	Iowa Darter *Etheostoma exile*	X	X
WP1	West Plum Creek	Longnose Sucker *Catostomus catostomus*		X

Table 14. Comparison of species detection by eDNA and by conventional methods at West Plum Creek on 10/14/2021.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
WP2	West Plum Creek	Creek Chub S*emotilus atromaculatus*	X	X
WP2	West Plum Creek	Longnose Dace *Rhinichthys cataractae*	X	X
WP2	West Plum Creek	Bigmouth Shiner *Notropis dorsalis*	X	X
WP2	West Plum Creek	Common Shiner *Luxilus cornutus*	X	X
WP2	West Plum Creek	Central Stoneroller *Campostoma anomalum*	X	X
WP2	West Plum Creek	Johnny Darter *Etheostoma nigrum*	X	X
WP2	West Plum Creek	White Sucker *Catostomus commersonii*	X	X
WP2	West Plum Creek	Brook Stickleback *Culaea inconstans*	X	X
WP2	West Plum Creek	Fathead Minnow *Pimephales promelas*	X	X
WP2	West Plum Creek	Longnose Sucker *Catostomus catostomus*		X

Table 15. Comparison of species detection by eDNA and by conventional methods at West Plum Creek on 10/13/2021.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
WP3	West Plum Creek	Longnose Dace *Rhinichthys cataractae*	X	X
WP3	West Plum Creek	Creek Chub S*emotilus atromaculatus*	X	X
WP3	West Plum Creek	Central Stoneroller *Campostoma anomalum*	X	X
WP3	West Plum Creek	Common Shiner *Luxilus cornutus*	X	X
WP3	West Plum Creek	Johnny Darter *Etheostoma nigrum*	X	X
WP3	West Plum Creek	Bigmouth Shiner *Notropis dorsalis*		X
WP3	West Plum Creek	White Sucker *Catostomus commersonii*	X	X
WP3	West Plum Creek	Brook Stickleback *Culaea inconstans*	X	X
WP3	West Plum Creek	Fathead Minnow *Pimephales promelas*	X	X
WP3	West Plum Creek	Iowa Darter *Etheostoma exile*	X	

Table 16. Comparison of species detection by eDNA and by conventional methods at East Plum Creek on 10/14/2021.

Site ID	Stream	Species	Detected by eDNA	Detected by conventional methods
EP1	East Plum Creek	Creek Chub S*emotilus atromaculatus*	X	X
EP1	East Plum Creek	Bigmouth Shiner *Notropis dorsalis*	X	X
EP1	East Plum Creek	Longnose Dace *Rhinichthys cataractae*	X	X
EP1	East Plum Creek	Brook Stickleback *Culaea inconstans*	X	X
EP1	East Plum Creek	White Sucker *Catostomus commersonii*	X	X
EP1	East Plum Creek	Fathead Minnow *Pimephales promelas*	X	X
EP1	East Plum Creek	Central Stoneroller *Campostoma anomalum*	X	
EP1	East Plum Creek	Common Shiner *Luxilus cornutus*	X	
EP1	East Plum Creek	Flathead Chub *Platygobio gracilis*	X	
EP1	East Plum Creek	Iowa Darter *Etheostoma exile*	X	

Table 17. Comparison of total species numbers detected by each method and unique species detections by each method per site.

Site ID	eDNA total	Conventional total	eDNA unique	Conventional unique
EP1	10	6	4	0
FC1	9	5	5	1
FC2	9	7	2	0
FC3	11	5	6	0
FC4	12	9	4	1
LH1	12	16	2	6
LP1	14	5	10	1
SP1	18	13	7	2
SP2	19	20	4	5
SV1	9	12	0	3
WP1	8	9	0	1
WP2	9	10	0	1
WP3	9	9	1	1
Mean	11.5	9.7	3.5	1.7

DISCUSSION

We detected a greater number of species per site using eDNA compared to electrofishing. Generally, the additional species detected corresponds with the species range, and few instances of species detections outside of historical range. We did experience some field contamination and the detections outside the range may be due to this. But overall, the patterns between the two methods correspond with eDNA providing additional resolution and sensitivity. As a pilot study, we identified that the field sampling protocol needs optimization to limit cross contamination.

In some cases of species detection disagreement between the sampling methods, such as the sites WP1 and WP2 (Table 13 and Table 14), the difference between eDNA and electrofishing was a single individual of a single species, *C. catostomus*. At these same sites both methods also detected the morphologically similar species, *C. commersonii*. It is possible that these disagreements are due to misidentification of the single individual *C. catosomus*. If it was a misidentification, then the agreement at these sites would be 100%. In cases such as these, eDNA can assist conventional methods by providing a check or confirmation of morphological identification.

The contamination issue requires resolution, but we still detected fish species diversity associated with the sampled watershed. It will require more bioinformatics work to assess the degree of influence the contamination had on the results. Future work will replicate the comparative site analysis, but with decontamination procedures occurring between sampling sites rather than between major watersheds. Our results demonstrate that eDNA can be a useful tool to collect species detection data more efficiently and cost-effectively than traditional methods. It is important to note that eDNA is a non-invasive

tool that avoids any potential injury to fish. Although there will always be a need for traditional sampling, eDNA provides a valuable alternative or complementary approach for obtaining information regarding species' distributions.

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PUBLICATIONS

Friebertshauser, R., T. Piaggio, M. Hopken, and R. Fitzpatrick. 2023. Environmental DNA Metabarcoding for Eastern Plains Native Fishes. Final Report to Colorado Parks and Wildlife (contract CT2021*3319). Fort Collins, Colorado.

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RESEARCH PRIORITY

Environmental DNA metabarcoding for crayfish in Colorado.

COLLABORATORS

- Dr. Toni Piaggio, Research Biologist, National Wildlife Research Center, USDA-APHIS-WS
- Ellyse Anderson, Biological Science Technician, National Wildlife Research Center, USDA-APHIS-WS
- Robert Walters, Invasive Species Program Manager, Colorado Parks and Wildlife
- Matt Bolerjack, Aquatic Nuisance Species Technician, Colorado Parks and Wildlife

OBJECTIVES

This project will use environmental DNA metabarcoding to detect native and potential invasive crayfish species. These results will provide baseline distribution maps for native species, as well as act as an early detection system for invasive species.

INTRODUCTION

Crayfish (Order: Decapoda) are an important component of many freshwater stream ecosystems as they play a role in nutrient cycling, act as a food source, and are indicators for water quality. Unfortunately, they are a taxa of conservation concern as the International Union for the Conservation of Nature (IUCN) lists 24% of all crayfish as threatened (Sibley et al. 2011). Colorado has eight native species of crayfish, which are located on the eastern slope (Walker 2007; Table 18). There are large information gaps for native crayfish species in Colorado, especially regarding their distribution. A leading mechanism for the decline of native crayfishes are invasive crayfish species. These species are often larger and more aggressive than native species, allowing them to outcompete native species for food resources. Invasive crayfish species have been translocated to act as a prey base for game fish and to consume unwanted aquatic plants (Carothers 1994). Nonnative species expansion has also potentially occurred due to transport for human consumption, and residual live crayfish being released into local watersheds. For effective management and conservation, a clearer understanding of native and nonnative crayfish distribution in Colorado is required.

Detecting small-bodied organisms in large river systems is difficult. Species monitoring using environmental DNA (eDNA) is a powerful new technique for wildlife detection that may improve the efficiency of these sampling efforts (Deiner et al. 2017; Piaggio 2021). The Smith Root aquatic eDNA sampling system was designed by a team of

molecular ecologists and engineers for high-throughput eDNA sample collection (Thomas et al. 2018). The system is optimized for sampling speed and replicability, while minimizing the risk of contamination. It is designed to sample larger volumes of water compared to other eDNA sampling methods, which reduces the risk of contamination among sampling sites (Thomas et al. 2018). Therefore, this system could easily be incorporated into CPW's sampling protocols and provide additional information regarding species distributions, especially for hard to detect species (Mariac et al. 2018).

Table 18. Crayfish species that will be sequenced and incorporated into the eDNA database.

Common name	Scientific name	Status
Louisiana Red Swamp Crawfish	*Procambarus clarkii*	Potential Invasive
Signal Crayfish	*Pacifastacus leniusculus*	Potential Invasive
Marbled Crayfish	*Procambaraus virginalis*	Potential Invasive
Southern White River Crayfish	*Procambarus zonangulus*	Potential Invasive
Rusty Crayfish	*Faxonius rusticus*	Invasive
Causey's Crayfish	*Orconectes causeyi*	Native
Ringed Crayfish	*Orconectes neglectus neglectus*	Native
Calico or Papershell Crayfish	*Faxonius immunis*	Native
Water Nymph or Kansas Pond Crayfish	*Orconectes nais*	Native
Northern Crayfish	*Orconectes virilis*	Native
Devil Crayfish	*Cambarus diogenes*	Native
Northern Clearwater Crayfish	*Orconectes propinquus*	Native
Southern Plains Crayfish	*Procambaraus simulans*	Native

METHODS

Species of interest (Table 1) will be sequenced and a genetic database will be developed. Tissue samples of species of interest will be collected via ongoing CPW field efforts, and, for invasive species that are not in Colorado, by being mailed samples from agencies in other states where those species occur. This approach of collecting our own samples and generating our own eDNA database is more robust and reliable than using outside databases, such as National Center for Biotechnology Information's GenBank. GenBank is known to contain errors, they may have low samples sizes within species, and even if one of our species is included, previous studies may not have amplified genetic fragments of interest to our study.

For environmental sampling we will implement a metabarcoding approach, which utilizes the high-throughput capability of next-generation sequencing platforms to sequence a single fragment of all amplifiable DNA from a sample. For an environmental sample, this means DNA from all species can be amplified in that sample. To extract DNA we will use the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). We have developed a metabarcoding assay in a prior study and we will use this one with primers targeted to fish and other vertebrates (MiFish; Shu et al. 2020), as well as others targeted to decapods (MiDeca; Komai et al. 2019). Library prep of field collected samples will be

conducted and then loaded on the NextSeq. NWRC developed database and data analysis pipeline. Statistics will include species relative abundance, occurrence per site, Shannon's diversity indices, and other parameters to provide insight into the presence or absence of the target invasive species and also biodiversity present within Colorado waterways. Field eDNA collection protocols will involve filtering three 2-L samples (at 1-L/min) and one, 2-L negative control with the backpack collection unit. Two-liter samples taken in triplicate should provide ample coverage of genetic material at a given site. After samples from a site are collected and stored, a field negative must additionally be collected in order to check for cross contamination.

The National Wildlife Research Center is a state of the art genomics facility completed in 2001 and being regularly updated. The Wildlife Genetics Lab under the direction of Dr. Toni Piaggio has a long history of developing and applying methods for non-invasive monitoring of wildlife species. This includes now extensive experience in developing environmental DNA (eDNA) assays and applying them to wildlife management successfully (Piaggio et al. 2014; Williams et al. 2016; Williams et al. 2017; Williams et al. 2018; Klymus et al 2020; Piaggio 2021). The Piaggio lab occupies four laboratories totaling ~1500 sq ft. Support space includes a glass wash and autoclave room, a media prep room, a PCR setup/library prep room, and other miscellaneous equipment rooms. Equipment dedicated to Dr. Piaggio's research includes instruments necessary for NGS library preparation and quality control. These include a Covaris m220 ultrasonicator, a QIAxcel, a Qiagen TissueLyser, QIAcube robotic liquid handling system, a Qubit for fluorometric quantitation, BioRad 9600 CFX qPCR machine, and an Illumina MiSeq. These instruments are all located in the Piaggio lab at the National Wildlife Research Center. They also have equipment necessary for conventional PCR and Sanger sequencing and fragment analysis. This includes a Thermo Fisher 3500 and 3500xl genetic analyzers and Eppendorf Mastercycler EP gradient Thermocyclers. The Piaggio Laboratory also has high-powered servers for the storage and analysis of NGS data. These include two analysis servers with 64x AMD Opteron 6378 processors and 512 Gb RAM each. The lab also maintains a storage server with 24x Intel Xeon E5-2620 v2 processors, 192 Gb RAM, and 24x4 Tb hard drives (96 Tb) arranged in a RAID 6 configuration, which automatically backs up the analysis servers every night.

Resulting data from environmental samples will be compared to the database generated from known Colorado species. This process is a highly technical and computationally expensive process but will yield information about target species presence and absence in sampled areas. This will also provide data that can be used in occupancy modeling approaches to estimate sampling efforts for other areas in Colorado. Finally, these data can be compared to other surveillance approaches for a more robust understanding of presence and distribution of target crayfish species.

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RESEARCH PRIORITY

Examination of hatchery stocking success of Plains Minnow *Hybognathus placitus*.

COLLABORATORS

- Ellyse Anderson, Aquatic Research Technician, Colorado Parks and Wildlife
- Karissa Coffield, Aquatic Research Technician, Colorado Parks and Wildlife
- Kalvin Lam, Aquatic Research Technician, Colorado Parks and Wildlife

OBJECTIVES

This project aims to assess the reproductive success of hatchery-released and translocated plains fishes by evaluating the production of early life stages. This will provide guidance as to whether continued stocking is necessary or if hatchery production would be better utilized in new locations.

EXECUTIVE SUMMARY

Between May 1, 2023 and September 1, 2023, three sites on the Arkansas River were sampled a total of 54 times resulting in 330 eggs and 495 larval or juvenile fish collected. In total, 15 species of fish were detected, including the state endangered Suckermouth Minnow and the species of concern Flathead Chub. Unfortunately, none of the eggs or fish collected were genetically identified as Plains Minnow. The number of eggs collected per volume of water sampled was highest during late May and early June. Maximum temperature and mean discharge both had significant effects on the number of eggs collected per volume of water sampled, with peak egg collection occurring when temperatures were approximately 22°C and discharge was approximately 14m^3/sec. The time of day that drift net samples were collected affected the number of eggs collected, with the highest egg catch occurring between 06:00 and 07:00. As the study season progressed, the number of larval and juvenile fish increased, and the day with the highest number of fish caught was August 24, 2023. Maximum temperature affected the number of fish caught in drift nets and Moore egg collectors, with spikes in maximum temperature often being followed by spikes in fish caught. The number of fish caught in drift nets was also affected by the time of day sampled, and the highest numbers of fish per volume of water sampled were observed between 06:00 and 07:30. Sampling efforts will continue in 2024.

INTRODUCTION

Hatcheries are an important tool used globally to supplement fish populations for multiple reasons, including ensuring recreational fishing remains sustainable and to assist in the conservation of vulnerable species (Richards 2023). In recent years, there has been an increase in hatcheries focusing on preserving genetic diversity of threatened and endangered species to minimize extinction risk (Fisch et al. 2015). Colorado Parks and Wildlife (CPW) has been utilizing fish hatcheries since 1881, with 19 hatcheries statewide raising over 30 species. The J. W. Mumma Native Aquatics Species Restoration Facility (NASRF) was established in 2000 to conserve rare native aquatic species. NASRF is leading the way in expanding the understanding of water quality, photoperiod, dietary needs, and appropriate spawning and habitat requirements unique to these rare species. NASRF implements a variety of methods to promote the conservation of 16 different fish species, including six plains fish species, involving captive propagation, genetic conservation, scientific research, public education, and awareness.

Great Plains fishes are an assemblage with a high number of at-risk or special status species (Worthington et al. 2018). There are 36 native plains fish species in the South Platte, Republican, and Arkansas River basins in Colorado and 12 of these are listed as endangered, threatened, or species of concern within the state. The reproductive strategy utilized by some of these species consists of a non-adhesive, semibuoyant egg that is released into the water column and then is passively transported downstream by the current (Moore 1944; Fausch and Bestgen 1997). This mode of reproduction is called pelagic-broadcast spawning and allows eggs to develop without being destroyed by shifting substrates during high flows (Fausch and Bestgen 1997). A primary threat to these pelagic spawning species and other Great Plains fishes is river fragmentation, which affects natural hydrology and habitat complexity (Cross and Moss 1987; Worthington et al. 2018). Barriers along a river, such as dams, decrease the abundance of pelagic spawning fish due to the need for longitudinal connectivity that allows eggs and larvae to develop (Perkin et al. 2015, Worthington et al. 2018). Flow variability is also necessary to keep eggs and larvae suspended in the water column to avoid suffocation and promote development, with larvae likely requiring a higher velocity due to their higher density (Worthington et al. 2018).

Hatchery-released fish are an important management tool for the conservation of Great Plains fishes in Colorado. Given the high conservation need of this assemblage and the amount of CPW staff required to rear fish in the hatchery, it is important to ensure that these fish are released in the most efficient locations possible. An assessment of hatchery stocking success, through the measure of species reproduction, will help determine if continued stocking in certain areas is required, or if future hatchery production would be better utilized in new locations to further expand the species range and increase the probability of persistence.

The purpose of this research is to provide information for the conservation and management of native fish populations. Although this project focuses on Plains Minnow *Hybognathus placitus*, the techniques can be applied to other Great Plains fishes in future research. We evaluated the stocking success attributed to reproduction in the wild of

hatchery-released Plains Minnows. We also collected data on abiotic factors to aid in future stocking or collection efforts, including discharge, temperature, net type, turbidity, flow, and both seasonal and diurnal time. Our research had two major elements: 1) a field examination for early life stages of wild progeny by capture of eggs and larval fish and 2) genetic identification of these early life stages to determine the presence of hatchery-released fish and wild-progeny adults. The results of this study will guide future stocking efforts and increase the probability of persistence of these fishes.

METHODS

Study Sites

Three sites on the Arkansas River were sampled in 2023: Melon Valley State Wildlife Area, Rocky Ford State Wildlife Area, and Oxbow State Wildlife Area (from upstream to downstream). Each study site is a location where Plains Minnows have been stocked by NASRF (Figure 2). Melon Valley was sampled throughout the entire field season, but both Rocky Ford and Oxbow were only sampled during the early and late portions of the season due to unusually high water levels in the Arkansas River that made sampling these sites unsafe. Temperature and discharge data were obtained from the United States Geological Survey gauge station number 07124000 at Las Animas, CO.

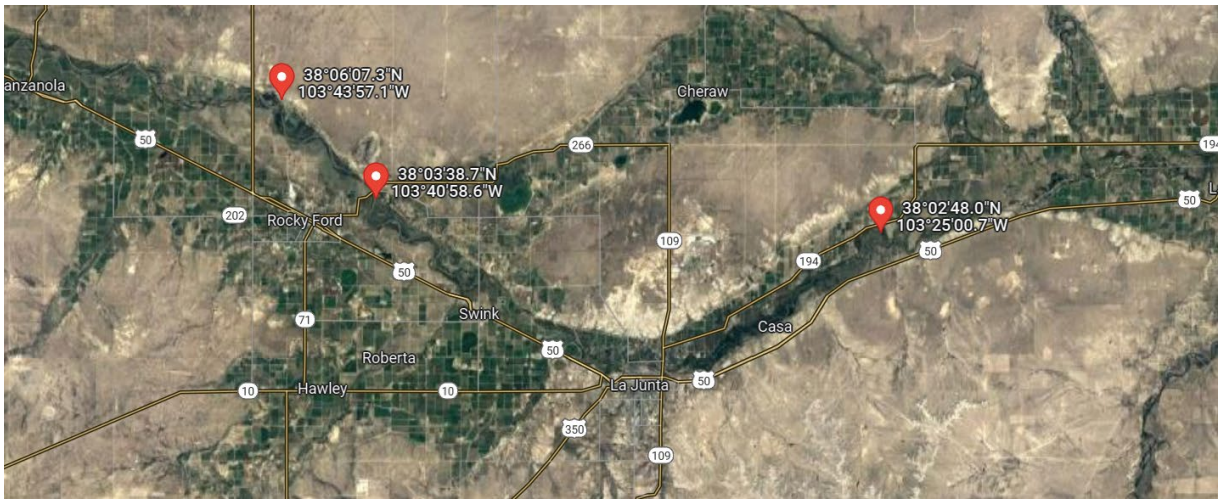

Figure 2. Collection sites for Plains Minnow early life history project on the Arkansas River. Sites from left to right are Melon Valley State Wildlife Area, Rocky Ford State Wildlife Area, and Oxbow State Wildlife Area.

Egg and Larval Fish Collections

Eggs were collected from the Arkansas River throughout the late spring and summer of 2023 using both drift nets and Moore egg collectors (Figures 3 and 4). Larval and juvenile fish were collected using a combination of drift nets, Moore egg collectors, seines, and dip nets. If fish were large enough to identify, they were measured and

released immediately. Fish that were too small to identify were preserved for genetic analysis or brought back to be raised to the CPW Salmonid Disease and Sport Fish Research Lab a larger size. One or two 500 µm mesh drift nets (0.76 m wide × 0.38 m high × 2.0 m long, tapered to an 11 cm opening) were deployed between 0600 and 1500 for varying amounts of time depending on the flow and turbidity of the water, ranging from 10 to 210 minutes. Drift nets were in main channel areas, as well as areas near vegetation, to increase the range of sampled habitat. Water velocity and drift net depth were recorded using a Hach wading rod, OTT MF Pro velocity sensor, and OTT MF Pro meter. Total volume of water sampled was calculated using sample duration, water velocity, and area of the submerged net. A subset of collected drift net samples were immediately preserved in 100% ethanol for picking within four days of collection to avoid deterioration. Eggs and larvae picked from preserved samples were counted and/or measured and stored in 70% ethanol vials for genetic testing (Figure 5). The remainder of drift net samples were picked on inverted Moore egg collectors in situ. Eggs and larvae found in situ were similarly preserved in 70% ethanol vials or kept alive and brought back to the CPW Salmonid Disease and Sport Fish Research Lab.

Figure 3. (A) Moore egg collector deployed and (B) three technicians monitoring for eggs and larvae with Moore egg collectors.

Moore egg collectors (0.43 m wide × 0.33 m high × 1.02 m long) were built following instructions put forth in Altenbach et al. (2000) and modified to more closely resemble those used by Haworth and Bestgen (2016). Moore egg collector deployment required sufficiently low water depth, as operation required the user to sit and monitor the collector for debris removal and egg collection (Figure 3). Submerged depth and water velocity were also recorded to calculate the total volume of water sampled. Egg and

larvae collection and preservation procedure was similar to that of drift net sampling. Dip netting and seining were performed along the shore near vegetation where juvenile and adult fish were observed to congregate, and specimens were identified and released or preserved in 70% ethanol.

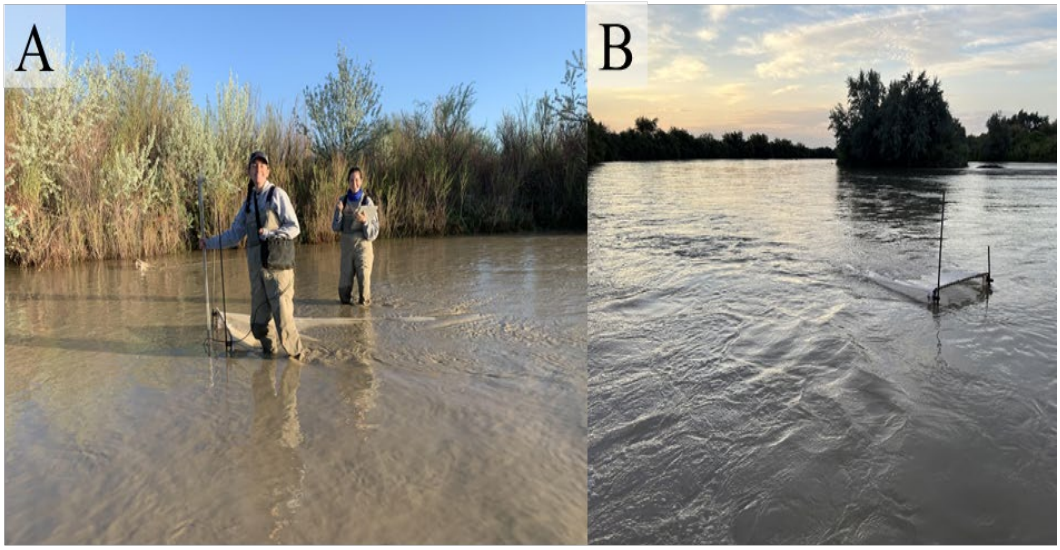

Figure 4. (A) Setting up a drift net while taking flow measurements and (B) a deployed drift net.

Genetic Collection and Analysis

A total of 79 vials were sent to Jonah Ventures for next-generation sequencing. According to the methods outlined by Jonah Ventures (2020), the first step in their process was to collect tissues from each vial using a sterile cotton swab, followed by genomic DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). After filtering and eluting the DNA, genes were PCR amplified using the MiFishU forward and reverse primer (Miya et al. 2015) and contained a 5' adaptor sequence to allow for indexing and Illumina sequencing. PCR amplification was performed in six separate replicates and each reaction was mixed according to the Promega PCR Mastermix specification (Promega, Madison, WI). After amplification, each reaction was visually inspected using a 2% agarose gel to determine amplicon size and PCR efficiency. Amplicons were then cleaned through a process of incubation and inactivation. In order to complete the sequencing library construct, a second round of PCR amplification was performed to include the final Illumina sequencing adapters and a sample specific index sequence. The final indexed amplicons were cleaned and normalized using SequalPrep Normalization Plates (Life Technologies, Carlsbad, CA) and pooled together. Sample library pools were sent for sequencing on an Illumina MiSeq

(San Diego, CA) located at the Texas A&M Agrilife Genomics and Bioinformatics Sequencing Core facility. Bioinformatics were performed and a consensus taxonomy was assigned using a custom best-hits algorithm and a reference database that includes sequences from GenBank and Jonah Ventures voucher sequence records.

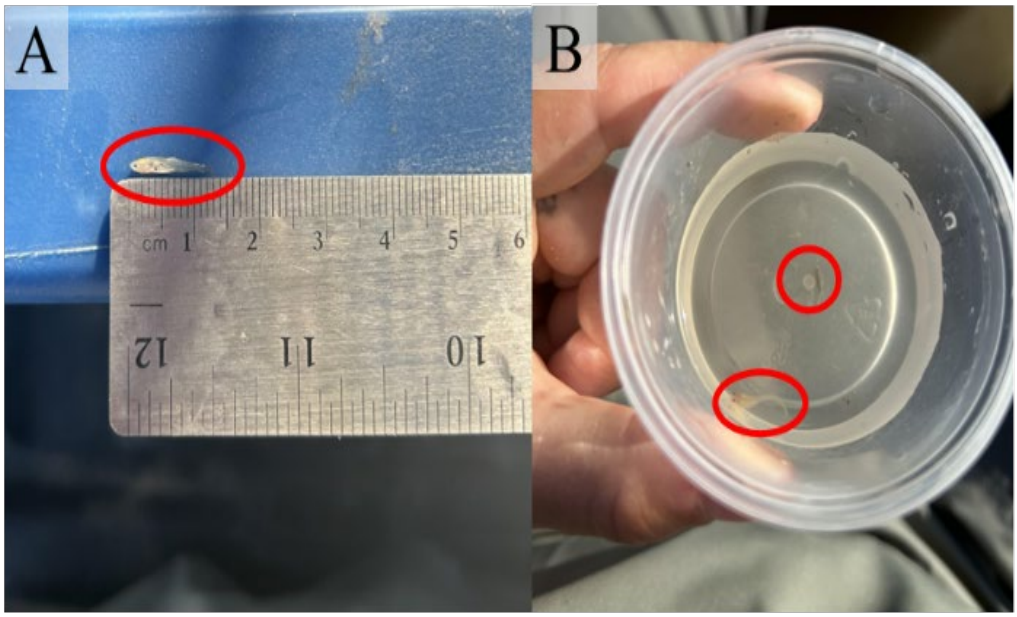

Figure 5. (A) Larval fish found in a preserved drift net sample and (B) an egg and larval fish collected using a Moore egg collector.

CPW Salmonid Disease and Sport Fish Research Lab Fish Holding

Unidentifiable larval and juvenile fish that were captured live in the field using Moore egg collectors, dip nets, or drift netting were transported to the CPW Salmonid Disease and Sport Fish Research Lab in Fort Collins. Fish were transferred from the field in one gallon water coolers with portable aerators. After returning to the CPW office, the fish were transferred to tanks where they could be reared to a size at which they could be identified. Fish that died in the facility before reaching an identifiable age were preserved for genetic testing in the same manner as other specimens collected in the field, and if possible, preserved in the vial corresponding to the same date and location of their live collection. In the lab, two 40 quart plastic storage containers were filled with 6 cm of water. A water heater was placed in the bottom of each one to create a water bath of approximately 25°C. A space heater was also run in the wet lab during the beginning and end of the season in order to maintain a stable air temperature. Beakers ranging from 500 mL to 2 L were used to separate fish collected by date and size. Three reservoir water buckets were also set up in the water bath for the purpose of having readily available at-temperature and dechlorinated water for water exchanges. All tanks and reservoirs had an

aerator. Water exchanges were performed twice weekly and all fish were fed following the water exchange. Aquarium fish pellets and dried daphnia were used as feed. A log documenting incoming fish, mortality, and the corresponding genetic vial was used in the facility.

Table 19. Research questions, statistical test used, and variables included to examine egg and larvae production in the Arkansas River, Colorado.

Question	Test utilized	Variables included
Is there a difference between the number of eggs/fish caught per volume of water sampled between Moore eggs collectors or drift nets?	Independent T-Test	Eggs/Fish Per Volume of water sampled and Net type used
Does mean/minimum/maximum daily temperature affect the number of eggs/fish caught per volume of water sampled?	ANOVA	Eggs/Fish Per Volume of water sampled and Mean/Maximum/Minimum Temperature
Does mean daily discharge affect the number of eggs/fish caught per volume of water sampled?	ANOVA	Eggs/Fish Per Volume of water sampled and Mean Daily Discharge
Does the time of day a sample was collected affect the number of eggs/fish caught per volume of water sampled?	ANOVA	Eggs/Fish Per Volume of water sampled and Time of Day sampled
Do the number of eggs/fish caught per volume of water sampled change across the sampling season?	ANOVA	Eggs/Fish Per Volume of water sampled and Date

Statistical analyses

All statistical analyses were completed using R version 4.3.1 (R Core Team 2023). An independent samples t-test was used to compare the eggs and fish per volume of water sampled caught with Moore egg collectors and drift nets. The independent t-test assumes that observations are independent and belong to only one group, there are no significant outliers, the data in each group is normally distributed, and the variances are homogeneous. All numerical values for eggs/fish per volume of water analyzed only belonged to either the group “Moore”, representing the Moore egg collectors, or the group “Drift”, representing the Drift nets. The data used were not normally distributed, but we utilized a data set that was large ($n>30$). The variances between the groups were homogenous.

Analysis of variance (ANOVA) tests were used to compare the eggs or fish per volume of water to different environmental variables. The p-value used in this study was anything less than or equal to 0.05 indicated significant results. ANOVA tests assume that observations are independent, groups have equal variance, and all data is normally distributed. The extreme outliers were not included in the analysis in order to meet assumptions. Although the data was not normally distributed, there was a large sample size used in this study (n>30). Research questions of interest for this study are provided in Table 19.

RESULTS

Egg collection summary

From May 1, 2023 to September 1, 2023, three sites were sampled along the Arkansas River a total of 54 times, resulting in a total of 330 eggs collected for genetic testing (Table 20). All egg and fish collections were standardized by the volume of water that was sampled when the organism was collected. Egg collection was highest in late May and Early June, with the highest amount of eggs caught on 5/25/2023 with a value of 0.031 eggs/m^3, but there was no significant difference across the season for the number of eggs collected per volume of water sampled (p=0.08; Figure 6). When possible, each site was sampled during a week, but during most of May and June, the region experienced high water levels and fast flows due to high amounts of precipitation, making sampling efforts difficult. During this time, sampling could only occur at Melon Valley and we were unable to use Moore egg collectors. This resulted in Melon Valley having the highest number of eggs per volume of water sampled (p=0.045; Table 20). Mean discharge significantly affects the number of eggs caught per volume of water sampled (p=0.034; Figure 7). The maximum daily temperature significantly affected the number of eggs caught in a day (p=0.006), but not the mean nor minimum temperatures (Figure 8). The time of day a drift net sample was collected had a significant effect on the number of eggs per volume of water captured, with early times having higher numbers of eggs per volume of water (p=0.0098; Figure 9). Although Moore collectors captured almost double the number of eggs per volume of water, there was no significant difference between the number of eggs collected per unit of volume between drift nets and Moore egg collectors (p=0.57; Table 21). Neither turbidity (p=0.94) nor flow (p=0.95) significantly affected the number of eggs captured per volume of water sampled.

Table 20. Summary of the number of visits, eggs collected, and fish collected at each sampling location. The number of egg and fish collections are standardized by the volume of water sampled.

Site	# Site visits	# Eggs	# Eggs/volume water (cm^3)	# Fish	# Fish/volume water (cm^3)
Melon Valley	35	286	0.00589	328	0.00676
Oxbow	9	17	0.00057	57	0.00191
Rocky Ford	10	27	0.00118	110	0.00481
Total	**54**	**330**	**0.00764**	**495**	**0.01348**

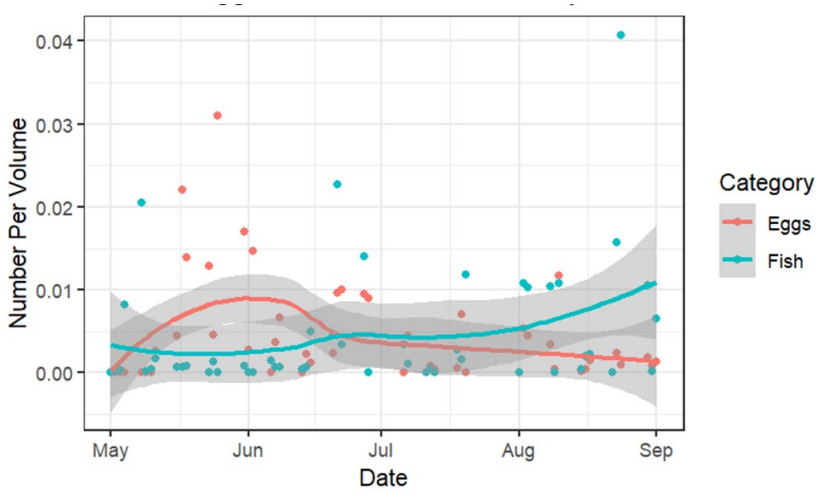

Figure 6. Number of eggs and fish caught per volume of water sampled across the study season, not including fish caught with dip nets.

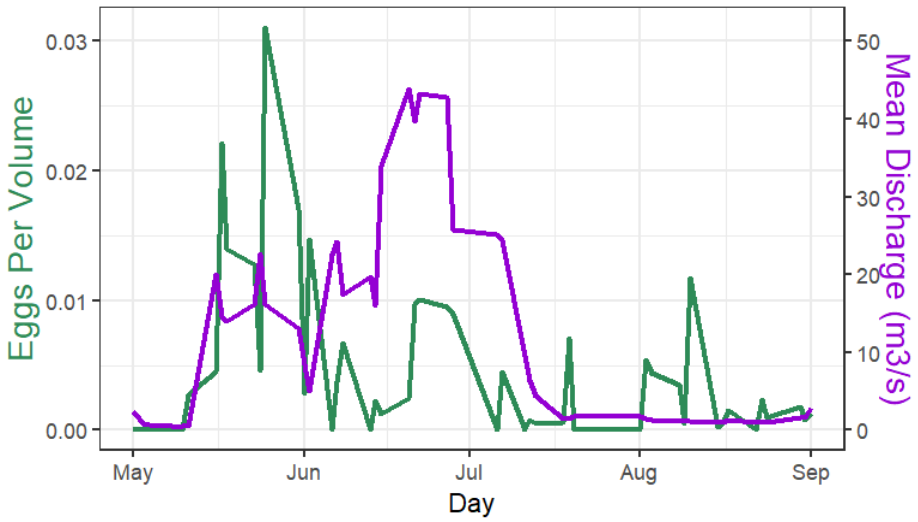

Figure 7. Number of eggs caught per volume of water sampled and the mean discharge across the study season.

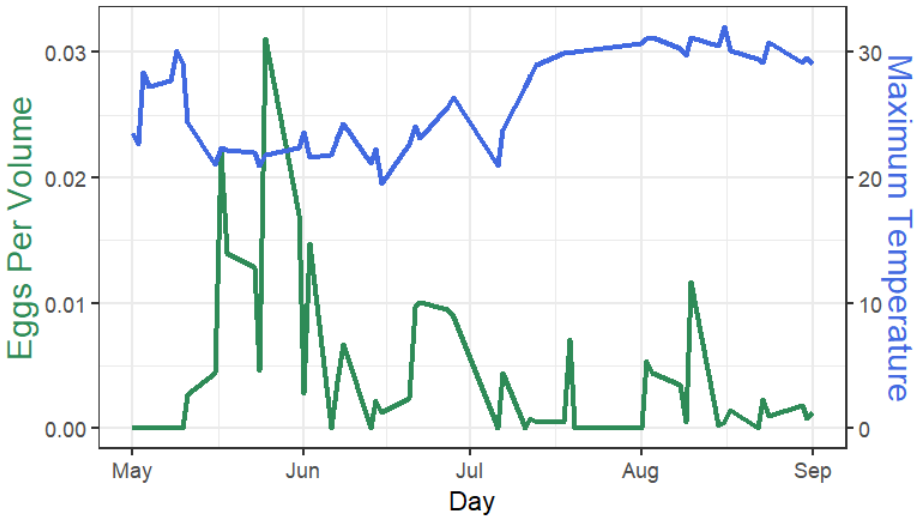

Figure 8. Number of eggs caught per volume of water sampled and the maximum temperature across the study season.

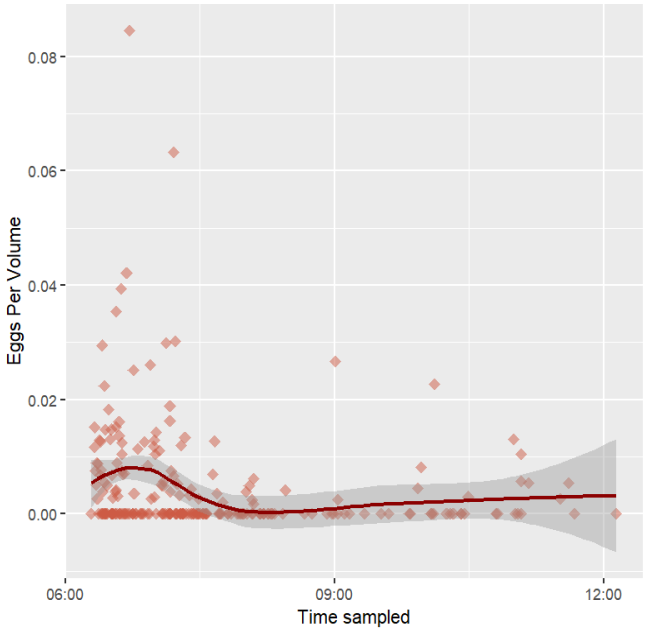

Figure 9. Number of eggs caught per volume of water sampled with a drift net over the course of a single sample day.

Larval fish collection summary

A total of 495 larval and juvenile fish were caught over the sampling season, 88 of which were captured using dip nets along the shore of the river (Table 20). This subset of dip-netted fish was not included in some analyses because we were unable to measure the volume of water sampled while dip-netting. The number of fish caught per volume of water sampled significantly increased as the season continued (p=0.034), with the highest amount of fish caught on 8/24/2023 with a value of 0.0407 (Figure 10). The maximum temperature significantly affected the number of fish caught in drift nets and Moore eggs collectors (p=0.03; Figure 10). The number of fish caught in drift nets was significantly affected by the time of day sampled, with earlier samples having significantly more fish (p=0.017; Fig 11). There is no significant difference between the number of fish caught per unit of volume water with drift nets or Moore egg collectors (p=0.104; Table 21).

Table 21. Comparison of eggs and fish caught using drift nets and Moore egg collectors.

Net Type	# Eggs	# Fish	Volume (m^3)	Eggs/Volume	Fish/Volume
Moore	105	23	18591	0.00612	0.0027
Drift	225	334	17153	0.00351	0.0035
Total	330	357	35645	0.00923	0.099

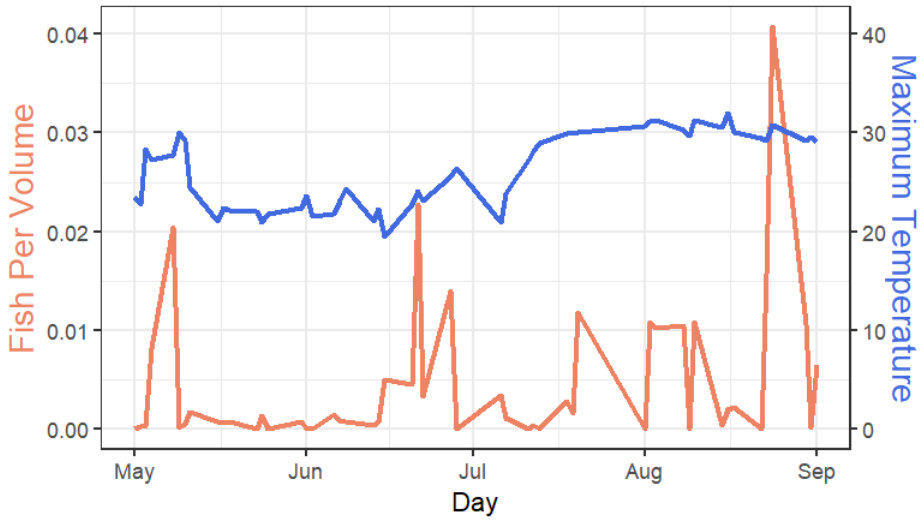

Figure 10. Number of fish caught per volume of water sampled and the maximum temperature across the study season.

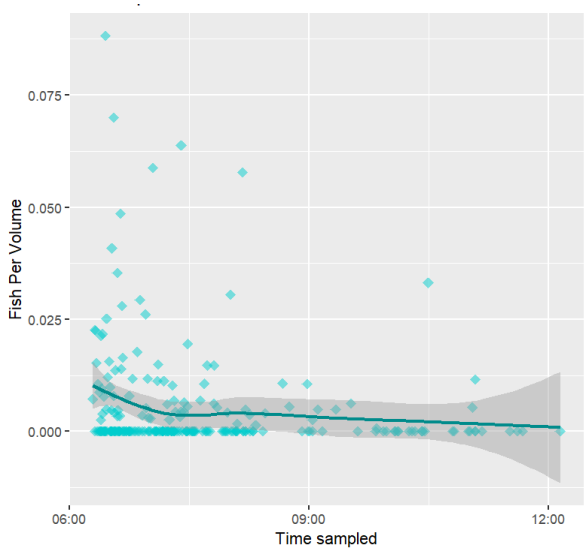

Figure 11. Number of fish caught per volume of water sampled with a drift net over the course of a single sample day.

CPW Salmonid Disease and Sport Fish Research Lab Summary

Over the 2023 field season, 35 larval or juvenile fish and 28 eggs were brought back from the field and housed in the lab. There was a 68% hatching rate, with 19 of the 28 eggs brought back from the field hatching during housing. All of the eggs or fish

housed in the lab were captured during either May or June except for one fish captured on July 13, 2023 (Table 22).

Table 22. Summary of the date fish or eggs were brought to the CPW Salmonid Disease and Sport Fish Research Lab and mortality of that group over the housing period.

Date	# Fish	# Eggs	Mortality
5/17/2023	1	0	1
5/18/2023	1	1	2
5/24/2023	1	0	0
5/25/2023	0	15	15
6/2/2023	9	0	8
6/8/2023	15	4	14
6/14/2023	0	1	1
6/20/2023	4	0	4
6/21/2023	3	7	10
7/13/2023	1	0	0
Total	35	28	55

Genetic summary

A total of 79 vials were sent to Jonah Ventures for genetic testing, but none of them were positive for Plains Minnow DNA. The species with the most sequence reads was Flathead Chub *Platygobio gracilis*, which is another pelagic spawning plains fish (Table 23). This species was found throughout the duration of the study season and characterized by variable peaks in sequence reads in a single sample vial (Figure 12). The fishes genetically identified at each sampling location also differed slightly (Table 24). There were also positive hits for Topeka Shiner *Notropis topeka*, but those results were removed as they were highly unlikely (more likely misclassified Sand Shiner *Notropis stramineus* DNA). This reiterates the importance of comparing eDNA results to a database of species that is likely to occur in your study area, and to check that database to be sure all species of interest are included in it.

Table 23. The absolute number of times each species' sequence was read by the sequencer.

Species	Sequence read count
Flathead Chub *Platygobio gracilis*	385,631
Red Shiner *Cyprinella lutrensis*	198,191
Walleye *Sander vitreus*	105,377
Bluegill *Lepomis macrochirus*	65,741
Channel Catfish *Ictalurus punctatus*	63,310
Plains Killifish *Fundulus kansae*	54,176
Fathead Minnow *Pimephales promelas*	45,362
Gizzard Shad *Dorosoma cepedianum*	25,424
White Sucker *Catostomus commersonii*	22,980
Suckermouth Minnow *Phenacobius mirabilis*	16,267
Yellow Bullhead *Ameiurus natalis*	7,555
Longnose Dace *Rhinichthys cataractae*	1,710
Green Sunfish *Lepomis cyanellus*	1,663
Western Mosquitofish *Gambusia affinis*	710
Brook Stickleback *Culaea inconstans*	34

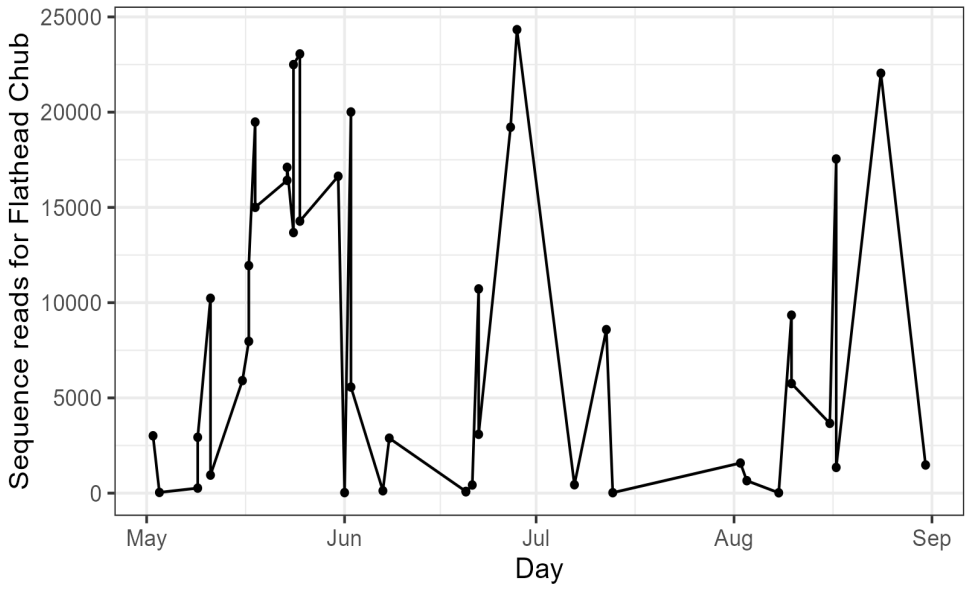

Figure 12. The number of times a Flathead Chub sequence was read over the duration of the study season.

Table 24. Species identified by site through eDNA sequencing.

Melon Valley SWA	Rocky Ford SWA	Oxbow SWA
White Sucker *Catostomus commersonii*	Red Shiner *Cyprinella lutrensis*	Yellow Bullhead *Ameiurus natalis*
Brook Stickleback *Culaea inconstans*	Gizzard Shad *Dorosoma cepedianum*	Red Shiner *Cyprinella lutrensis*
Red Shiner *Cyprinella lutrensis*	Plains Killifish *Fundulus kansae*	Plains Killifish *Fundulus kansae*
Gizzard Shad *Dorosoma cepedianum*	Western Mosquitofish *Gambusia affinis*	Channel Catfish *Ictalurus punctatus*
Plains Killifish *Fundulus kansae*	Channel Catfish *Ictalurus punctatus*	Green Sunfish *Lepomis cyanellus*
Channel Catfish *Ictalurus punctatus*	Green Sunfish *Lepomis cyanellus*	Bluegill *Lepomis macrochirus*
Green Sunfish *Lepomis cyanellus*	Bluegill *Lepomis macrochirus*	Suckermouth Minnow *Phenacobius mirabilis*
Bluegill *Lepomis macrochirus*	Fathead Minnow *Pimephales promelas*	Flathead Chub *Platygobio gracilis*
Suckermouth Minnow *Phenacobius mirabilis*	Flathead Chub *Platygobio gracilis*	Walleye *Sander vitreus*
Fathead Minnow *Pimephales promelas*		
Flathead Chub *Platygobio gracilis*		
Longnose Dace *Rhinichthys cataractae*		
Walleye *Sander vitreus*		

DISCUSSION

Data analysis and interpretation of 2023 sampling efforts are ongoing. Although no Plains Minnow DNA was detected, an additional 15 species were detected. This included the state endangered Suckermouth Minnow *Phenacobius mirabilis*, which was a previously hatchery-released species. Additionally, DNA of the pelagic spawning and species of concern Flathead Chub was collected throughout the study site and had the highest sequence read count of any species.

Future sampling needs to continue to focus on early morning hours as this resulted in the highest number of both eggs, and larvae/juvenile fish collected. The most eggs per volume of water sampled were collected during late May, with other abiotic factors such as mean discharge, maximum daily water temperature, and time of day also being significantly correlated with egg collection (Figures 5–8). Egg collection was highest when discharge was approximately 14 cms and when maximum daily temperatures were approximately 22℃ (Figures 7 and 8). Significant increases in egg collection during periods of high discharge correspond with findings by Taylor and Miller of increased reproductive activity coinciding with pulses of discharge (1990). Although these factors aid in the understanding of plains fish ecology, timing of peak egg production is likely to slightly fluctuate between years as a result of seasonal flow regimes and weather (Haworth and Bestgen 2016).

When comparing sites, eggs collected per volume of water was significantly higher at Melon Valley SWA. This could be attributed to the fact that it was sampled the most during peak egg production of the 2023 season because of unusually high water levels during the early months of summer, which made sampling at the other two sites unsafe. Future sampling should try to balance sampling efforts throughout the study area,

if those areas are safe for crews to sample. Moore egg collectors caught nearly twice as many eggs per volume of water sampled as drift nets while drift nets caught slightly more larval fish per volume of water sampled, so multiple gear types should continue to be used to increase detection probability of eggs and larvae. Due to the high flows of 2023 reducing detection probability of early life stages and reducing areas that could be safely sampled, sampling will continue in 2024.

ACKNOWLEDGMENTS

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Appendix I. Sampling information for each 2023 Plains Minnow early life history sampling event on the Arkansas River, Colorado.

Date	Site	# Eggs	# Fish	Mean Temp (Celsius)	Mean Discharge (m^3/s)	Volume (m^3)	Flow (m/s)	Turbidity (cm)
5/1/2023	Oxbow	0	0	17.51	2.42	345.26	0.42	N/A
5/2/2023	Oxbow/Rocky Ford	0	1	16.98	1.43	4079.56	0.40	N/A
5/3/2023	Melon Valley/ Rocky Ford	0	1	19.04	0.68	3428.18	0.47	N/A
5/4/2023	Melon Valley	0	9	19.29	0.65	1101.18	0.37	N/A
5/8/2023	Melon Valley	0	36	18.43	1.14	1755.80	0.49	N/A
5/9/2023	Oxbow	0	1	19.95	0.74	5181.75	0.37	N/A
5/10/2023	Rocky Ford	0	2	18.67	0.53	4113.47	0.47	N/A
5/11/2023	Melon Valley	3	2	17.28	0.49	1114.57	0.43	N/A
5/16/2023	Melon Valley	6	1	18.11	0.46	1344.41	0.34	N/A
5/17/2023	Melon Valley	32	1	20.04	0.48	1447.55	0.27	N/A
5/18/2023	Melon Valley	18	1	20.05	0.49	1292.00	0.25	N/A
5/23/2023	Melon Valley	26	0	20.13	1.08	2029.11	0.32	N/A
5/24/2023	Melon Valley	10	3	19.40	7.68	2163.65	0.31	N/A
5/25/2023	Melon Valley	49	0	19.40	27.52	1578.02	0.53	N/A
5/31/2023	Melon Valley	22	6	20.66	28.40	1291.11	0.78	N/A
6/1/2023	Melon Valley	4	8	20.70	19.99	1435.45	0.72	N/A
6/2/2023	Melon Valley	10	0	19.31	14.57	679.26	0.65	N/A
6/6/2023	Melon Valley	0	11	19.45	14.02	671.32	0.22	6.2
6/7/2023	Melon Valley	5	7	20.83	17.66	1337.19	0.44	5.7
6/8/2023	Melon Valley	9	1	21.74	12.30	1343.10	0.52	3.25
6/13/2023	Melon Valley	0	1	19.28	12.65	2493.45	0.56	7.2
6/14/2023	Melon Valley	3	1	19.39	10.27	1324.08	0.50	1.5
6/15/2023	Melon Valley	1	4	18.74	16.21	802.10	0.47	2.5
6/20/2023	Melon Valley	8	15	21.40	22.60	3288.52	0.57	3.75

6/21/2023	Melon Valley	20	50	22.11	16.12	2069.75	0.66	4.9
6/22/2023	Melon Valley	12	4	22.29	21.31	1190.06	0.60	5.7
6/27/2023	Melon Valley	17	25	24.14	20.13	1783.81	0.50	5.7
6/28/2023	Melon Valley	1	0	24.28	19.25	111.68	0.19	6.2
7/6/2023	Melon Valley	0	2	18.68	18.53	584.36	0.70	4.5
7/7/2023	Melon Valley	4	1	21.94	15.45	896.49	0.77	7.3
7/11/2023	Melon Valley	0	0	23.94	13.08	1806.49	0.54	3.0
7/12/2023	Rocky Ford	2	1	25.18	8.43	2580.72	0.57	4.8
7/13/2023	Melon Valley	1	0	24.64	5.01	1996.43	0.53	4.0
7/18/2023	Rocky Ford	1	5	24.15	8.27	1782.44	0.67	5.3
7/19/2023	Oxbow	9	2	23.87	11.03	1267.35	0.47	20.0
7/20/2023	Melon Valley	0	10	23.27	15.08	844.76	0.62	7.5
8/1/2023	Oxbow	0	4	24.42	22.57	3756.67	0.45	44.0
8/2/2023	Rocky Ford	7	14	24.59	24.21	1294.99	0.55	2.25
8/3/2023	Melon Valley	6	20	24.71	17.34	1364.55	0.67	2.0
8/8/2023	Rocky Ford	5	15	23.38	10.14	1433.41	0.69	4.0
8/9/2023	Oxbow	1	0	23.40	8.64	2075.78	0.44	9.8
8/10/2023	Melon Valley	13	12	24.04	8.93	1109.14	0.75	5.3
8/15/2023	Oxbow	1	25	22.92	14.19	4689.39	0.47	18.0
8/16/2023	Rocky Ford	1	4	24.30	19.65	1968.91	0.52	9.3
8/17/2023	Melon Valley	2	3	24.14	16.05	1327.12	0.58	8.8
8/22/2023	Oxbow	0	23	23.11	33.95	3291.67	0.34	> 95.0
8/23/2023	Rocky Ford	6	40	23.07	45.43	2539.83	0.56	13.8
8/24/2023	Melon Valley	2	83	23.59	43.43	2039.09	0.51	13.3
8/30/2023	Rocky Ford	5	29	23.30	45.62	2736.35	0.49	6.7
8/31/2023	Oxbow	6	1	23.43	46.09	7485.19	0.62	3.8
9/1/2023	Melon Valley	2	10	24.40	43.86	1535.10	0.64	5.5

RESEARCH PRIORITY

Evaluation of the Longrie-Fecteau fish passage structure and potentially use this structure as a relatively low cost template for other plains fish barriers.

CITATION

Fitzpatrick, R. M., D. L. Longie, R. J. Friebertshauser, and H. P. Foutz. 2023. Evaluation of a prefabricated fish passage design for Great Plains fishes. Fishes 8(8), 403. https://doi.org/10.3390/fishes8080403

ABSTRACT

Connectivity is critical for stream fish persistence, and fish passage structures are a useful conservation tool to reconnect fragmented systems. The design of fish passage structures is a tradeoff between the area available for construction, slope, and costs associated with the structure. The Longrie–Fecteau fish passage structure was designed to be modular, adjustable to barrier-specific needs, and to have a low slope (2%) to pass small-bodied fishes. We evaluated fish passage through this structure in Fountain Creek, Colorado, USA, via a PIT tag mark–recapture study. We documented four native Great Plains fish species successfully ascending the passage structure, with most passage occurring at night. We estimated a 3% probability of a released fish entering the structure, then 89% and 99% passage to the midpoint and exit of the 123 m structure, respectively. Low entrance efficiency was due to low recapture probability of small-bodied study organisms in a relatively large system, and the low percentage of space of the entryway on this barrier (<3% of the length of the barrier). Fish that entered the structure ascended quickly, with a median time for successful ascent of 19 min and a minimum time of 6 min. The Longrie–Fecteau fish passage structure is a conservation tool that may broaden the adoption of fish passage structures for small-bodied fishes due to its modularity and low slope.

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RESEARCH PRIORITY: The effects of abiotic enrichment and predator training on the behavior, morphology, and survival of a species of conservation concern.

CITATION

Kopack, C. J., E. R. Fetherman, D. E. Broder, **R. M. Fitzpatrick**, and L. M. Angeloni. 2023. The effects of environmental enrichment on behavior, morphology, and survival of a species of conservation concern. Conservation Science and Practice. https://doi.org/10.1111/csp2.12999

ABSTRACT

Conservation practitioners often rely on captive breeding programs to supplement wild populations at risk of extinction. While supplementation has been successful for some taxa, the success of using hatchery fish to supplement wild populations is severely impacted by predation. Elevated predation on hatchery fish may arise because hatchery environments often differ from wild environments and constrain the ability of hatchery fish to adapt to the environments in which they are released. We assessed the effects of abiotic enrichment on the expression of behavioral and morphological phenotypes across three populations of a species of conservation concern, the Arkansas darter (*Etheostoma cragini*). We also used a factorial approach to assess whether abiotic enrichment and predator training increase survival during encounters with a novel predator. We found that abiotic enrichment affected ecomorphological attributes associated with fins; generally, measures of the caudal and pectoral fin decreased in the treatment group compared to the control treatment. Behaviorally, darters reared with abiotic enrichment increased feeding and decreased movement compared to the control group. Importantly, we found that in combination with predator training, abiotic enrichment increased the probability of surviving first encounters with a predator. We recommend conservation practitioners incorporate abiotic enrichment and predator training in hatchery programs. Captive breeding programs are used to supplement wild populations at risk of extinction, but hatchery-reared fish often do not survive after release. Using the threatened Arkansas Darter, we show that abiotic enrichment and predator training of hatchery populations impact behavior and morphology and increase the probability of surviving first encounters with a non-native predator.

ADDITIONAL PUBLICATION

Kopack, C. J., E. R. Fetherman, D. E. Broder, R. M. Fitzpatrick, and L. M. Angeloni. 2023. Assessing antipredator behavior and the potential to enhance it in a species of conservation concern. North American Journal of Aquaculture 85(2):136–145. https://doi.org/10.1002/naaq.10281

RESEARCH PRIORITY: Effects of slope on small-bodied fish passage in an experimental rock ramp fishway

CITATION

Swarr, T. R., C. A. Myrick, and **R. M. Fitzpatrick**. *Under review*. Effects of slope on small-bodied fish passage success in an experimental rock ramp fishway. Transactions of the American Fisheries Society.

ABSTRACT

We used an experimental rock ramp fishway to evaluate the effects of fishway slope (2, 4, 6, 8, and 10%) and length (up to 6.1 m) on the passage success of three small-bodied fishes representative of the fauna of interior rivers in the United States: Flathead Chub *Platygobio gracilis*, Stonecat *Noturus flavus*, and Arkansas Darter *Etheostoma cragini*. The probability of passage success (Cormack–Jolly–Seber model) was highest over shorter distances and at lower slopes for all species. Probability of passage success (PPS) was highest for Flathead Chub, followed by Stonecat, and then Arkansas Darter. Flathead Chub had a PPS of 1.0 for ascending the full 6.1 m at 2, 4, and 6% slopes, dropping to 0.96 for a 4.06 m × 8% slope fishway; they could not ascend more than 4.06 m at 10% slope. Stonecat PPS was 1.0 for the full fishway at 2 and 4%, decreasing to 0.83 for a 4.06 m × 6% fishway, with no passage predicted for 6.1 m × 8% or 4.06 m × 10% length × slope combinations. Arkansas Darter PPS ranged from 0.54 for a 4.06 m × 2% slope fishway to 0.43 for a 2.03 m × 4% slope fishway and fell to 0 for all lengths at 10% and for combinations exceeding 6.1 × 6% and 4.06 m × 8%. This study provides valuable information on slope and length combinations that maximize the PPS of these small-bodied Great Plains fishes.

ADDITIONAL PUBLICATIONS

Swarr, T. R., C. A. Myrick, and **R. M. Fitzpatrick**. 2021. Tag retention in and effects of passive integrated transponder tagging on survival and swimming performance of a small-bodied darter. Journal of Fish Biology, 1–10. https://doi.org/10.1111/jfb.14984

Swarr, T. R., **R. M. Fitzpatrick**, and C. A. Myrick. 2023. Design, construction, and preliminary hydraulic evaluation of a model rock ramp fishway. North American Journal of Fisheries Management. https://doi.org/10.1002/nafm.10902