Final Performance Report

Office of
CONSERVATION
INVESTMENT

Office of Conservation Investment

U.S. Fish and Wildlife Service

OK T-129-R-1 Genetic Identification and Estimation of Population Demographics for Oklahoma Buffalofish (Ictiobus bubalus, I. cyprinellus, I. niger)

Final Performance Report Generated 01/26/2026

Performance Report Approval Status:

Awaiting Federal Approval

Recipient:

OKLAHOMA DEPARTMENT OF WILDLIFE

Recipient Grant ID:

Federal Award Number:

F22AF02999

Funding Program(s) Name:

SWG Implementation

Federal Award Start and End Date:

Jan 01, 2023 to Jun 30, 2025

Performance Reporting Period:

Jan 01, 2025 to Jun 30, 2025

Federal Award Recipient Contact(s):

Andrea Crews

Federal Award Specialist(s):

Joshua Cocke

TRACS Group

Oklahoma Department of Wildlife Conservation

Type of Performance Report:

Final Performance Report

Public Description:

Using genetic tools (genome-side SNP data) developed for three native, nongame Oklahoma species of buffalofish (Bigmouth Buffalo *Ictiobus cyprinellus*, Black Buffalo *I. niger*, and Smallmouth Buffalo *I. bubalus*), this project will provide genetically determined individual

species identifications, assess genetic diversity within and among populations of buffalofish, identify hybrid individuals and hybrid classes, assess deeper levels of hybridization among the three species, estimate effective population size for contemporary populations, and infer recent demographic trends for each population. These data and analyses will assist Oklahoma Department of Wildlife Conservation biologists in constructing age, size, sex structure characterization, and provide insight into population and demographic dynamics for the benefit of sustainable management.

Federal Award Accomplishments				
Strategy	**Proposed Objective**	**Activity**	**Unit of Measure - Proposed**	**Unit of Measure - Reported**
Research, Survey, Data Collection and Analysis	Conduct investigations (legacy)	Fish and wildlife species data acquisition and analysis (legacy)	1 Investigations	1 Investigations

Table of Contents - Project Statements

Project Statement: OK T-129-R-1 Genetic Identification and Estimation of Population Demographics for Oklahoma Buffalofish (Ictiobus bubalus, I. cyprinellus, I. niger)
Project Statement Approval Status: Final Approved

Objective Name: Objective 1: Complete 1 investigation by December 31, 2024

Strategy: Research, Survey, Data Collection and Analysis
Proposed Objective: Conduct investigations (legacy)
Pertains to R3: No

Activity Performed: Fish and wildlife species data acquisition and analysis (legacy)
# of Investigations: 1
Principal Investigator: Guinevere O.U. Wogan
Geographic Location:

- General Location: Oklahoma
- Includes Marine Federal Waters: No
- Detailed Location:
- Location Description:

  The activities of this proposal are restricted to genetic laboratory work, with samples provided by Oklahoma Department of Wildlife Conservation biologists. Genetic work will take place on the Oklahoma State University Campus in Stillwater, OK Payne County.

Activity Report Comments

* Totals to date represents a cumulative total of all periods of performance and may exceed the objective.

Objective Report	
Period of Performance	**# of Investigations**
Jan 1, 2023 to Dec 31, 2023	
Jan 1, 2024 to Dec 31, 2024	
Jan 1, 2025 to Jun 30, 2025	1
Totals to Date*	1

Species Tags

Species Tags
smallmouth buffalo *Ictiobus bubalus*
bigmouth buffalo *Ictiobus cyprinellus*
black buffalo *Ictiobus niger*

Activity Performed Attachments

Note: Some attachments listed here may not appear in the Appendix due to file incompatibility. All attachments can be accessed using the links below.

Descriptive Name	Field Tags	Attachment Type
No Files Attached		

Performance Reporting Questionnaire

1. What progress has been made towards completing the objective(s) of the project?

Overview 2023 *: Buffalofishes (Ictiobus cyprinellus, I. bubalus, I. niger) are three species of river- or lake-dwelling fish distributed in North America including Oklahoma that were previously believed to live for ~15 years, but recent discovery shows that I. cyprinellus individuals can live for over a century (Lackmann et al. 2019, Lackmann et al. 2021). Longevity of buffalofishes in Oklahoma is less extreme, however, I. bubalus has been shown to exceed 60 years (Snow, et al. 2020). Their longevity in combination with episodic recruitment suggests that population dynamics should be updated to ensure that effective management practices are in place. In Oklahoma, these fish are targets of recreational and tournament bowfishing, with large individuals that are likely older reproductive females being the prime targets (Scarnecchia and Schooley 2020). Furthermore, morphological identifications of smallmouth buffalo and black buffalo are confounded by hybridization (Stevenson 1964, Johnson and Minckley 1969), making morphological identification of these two species difficult (Schooley et al. 2023). Efforts to understand the aging and population dynamics of this fish will help us understand how to better manage and protect them if needed, as well provide us novel information on aging (Barnett et al. 2017, Love et al. 2019, Lackmann et al. 2021). The primary objective of this grant is to use genetic methods to identify species and detect hybridization. These insights will complement morphologically determined identifications using traditional morphological traits and a depth index (Di = SL/BD) (Hubbs et al. 2008) for the three species of buffalofishes (Bart et al. 2010, Schooley et al. 2023). With these data, population age structures for each species of buffalofish will be calculated by ODWC biologists to assess the impacts of current management practices on these long-lived episodically reproducing non&#x2;game fish. A combination of otolith aging (ODWC) and telomere lengths (OKState) are being used to determine the individual ages of each fish.*

Progress to Date 2023: *fin clip samples from buffalofishes were taken by ODWC biologists from 8 locations (n=730) across Oklahoma and stored in 100% ETOH until delivered to OK State where they were kept refrigerated until extraction. Grad student K. Mapes joined on two occasions for the collection of samples and collected additional tissues for later RNA expression assays including internal abdominal swabs, testes/gonadal tissues, and eggs, which were stored in cryotubes on dry ice and immediately transported back to the OK State laboratory where they were stored in a -20 freezer. We developed and optimized protocols for DNA extraction and PCR amplification for genetic barcoding for the cytochrome-b mitochondrial gene. Optimization of our protocols has resulted in high-yield DNA extracts from fin clip samples, we have settled on using a long 20-24 hour tissue digest on rotating tube mixers followed by a spin-column based extraction protocol. Using a qubit 4 fluorometer with the broad range assay we quantified DNA concentrations, and had very high-yield, high-quality results, with some samples exceeding the readable concentration of the assay. These results are more than sufficient for our goals of generating PCR-based barcoding and autosomal next generation sequencing. We have extracted 157 samples and have an additional set of recently acquired samples to extract. The*

cytochrome-b mitochondrial gene (maternally inherited) is commonly used for genetic determination of species identity. We have optimized our mitochondrial barcoding experiments by testing a series of primer combinations used in prior studies (Harris et al. 2002, Bagley et al. 2018) for three gene regions (cytochrome-b, COI, ND2), and have experimentally determined a set of PCR-amplification conditions that yield clean sequence reads for cytochrome-b using cytb514L and cytbH1591 primer combination with an annealing temperature of 50°C. Each sample was visually checked for a distinct amplification band of the correct size using agarose gel electrophoresis, cleaned to remove excess reagents, and then cycle sequenced using BigDye (ABI) prior to Sanger sequencing. Each sample was bidirectionally sequenced to ensure that forward and reverse reads were in agreement for each basepair. Sequences were processed and aligned using Sequencher Software (GeneCodes). Using the cytochrome-b gene we used BLAST (Camacho et al. 2009) searches through Genbank to check the species identity of 40 cyt-b sequences. Thus far we have identified individuals of two species of buffalo fish- bigmouth (I. cyprinellus) and smallmouth (I. bubalus). Many of the genetic and morphological identifications (ODWC) and Di-based (ODWC) assignments were in agreement. However, there are several instances where the morphological and Di-based assignments differed, and some instances where the genetic identity and morphologically and Di-based assignments disagreed. We have not definitively assigned any individuals to black buffalo (I. niger) using mitochondrial barcoding, however, two sequences were ambiguously assigned to this species. We will repeat the reactions for these two individuals to evaluate the assignment with additional data. We have a substantial number of samples for which barcoding data have not been generated. We are actively working on completing barcoding benchwork so that we may help refine species identities for the remaining samples. Since hybridization may be rampant among buffalofishes, mitochondrial DNA provides some insight into species identity, but due to its maternal inheritance may be discordant from the biparentally inherited autosomal genome. This phenomena is well documented in a variety of organisms, and discordance between mitochondrial genes and autosomal genes is commonly used to identify hybridized individuals in a population of interest (Wiens and Penkrot 2002, Pidancier et al. 2006, Leaché and Cole 2007, Hinojosa et al. 2019). In order to generate the autosomal genomic data we have elected to use genome-wide SNP data which we will generate through ddRAD library preps (Peterson et al. 2012). The ddRAD approach can generate many thousands of SNPs from throughout the genome for each individual which can then be used to infer population structure, species identity, hybridization, and effective population size. We have started the library preparation for generating the autosomal markers. In this library preparation process, DNA is digested with two restriction enzymes that perform double cuts which are thenindividually labelled with adapters and pooled for sequencing. We will generate ddRAD data for all of the samples that we currently have in hand. Our goal is to submit our first lane of sequencing by the end of April 2024, and to perform a second set of library preps and sequencing for any additional samples we receive by November 2024. In addition to the core of our work, the graduate student at OK State (K. Mapes) has been working to optimize a quantitative PCR based assay to measure the relative length of telomeres of each individual fish by comparing telomere copies to a single copy gene (in this case actin) to

calculate a T/S ratio which provides an estimate of telomere length (Blackburn 1991, Cawthon 2002). We will evaluate telomere length estimated age in combination with otolith estimated age to determine if telomere lengths are reliable biomarkers of age in these three species of fish.

Progress to Date 2024: *fin clip samples from buffalofishes were taken by ODWC biologists from 8 locations (n=730) across Oklahoma and stored in 100% ETOH until delivered to OK State where they were kept refrigerated until extraction. Grad student K. Mapes joined on two occasions for the collection of samples and collected additional tissues for later RNA expression assays including internal abdominal swabs, testes/gonadal tissues, and eggs, which were stored in cryotubes on dry ice and immediately transported back to the OK State laboratory where they were stored in a -20 freezer. We developed and optimized protocols for DNA extraction and PCR amplification for genetic barcoding for the cytochrome-b mitochondrial gene. Optimization of our protocols has resulted in high-yield DNA extracts from fin clip samples, we have settled on using a long 20-24 hour tissue digest on rotating tube mixers followed by a spin-column based extraction protocol. Using a qubit 4 fluorometer with the broad range assay we quantified DNA concentrations, and had very high-yield, high-quality results, with some samples exceeding the readable concentrationof the assay. These results are more than sufficient for our goals of generating PCR-based barcoding and autosomal next generation sequencing. We have extracted 157 samples and have an additional set of recently acquired samples to extract. The cytochrome-b mitochondrial gene (maternally inherited) is commonly used for genetic determination of species identity. We have optimized our mitochondrial barcoding experiments by testing a series of primer combinations used in prior studies (Harris et al. 2002, Bagley et al. 2018) for three gene regions (cytochrome-b, COI, ND2), and have experimentally determined a set of PCR-amplification conditions that yield clean sequence reads for cytochrome-b using cytb514L and cytbH1591 primer combination with an annealing temperature of 50°C. Each sample was visually checked for a distinct amplification band of the correct size using agarose gel electrophoresis, cleaned to remove excess reagents, and then cycle sequenced using BigDye (ABI) prior to Sanger sequencing. Each sample was bidirectionally sequenced to ensure that forward and reverse reads were in agreement for each basepair. Sequences were processed and aligned using Sequencher Software (GeneCodes). Using the cytochrome-b gene we used BLAST (Camacho et al. 2009) searches through Genbank to check the species identity of 40 cyt-b sequences. Thus far we have identified individuals of two species of buffalo fish- bigmouth (I. cyprinellus) and smallmouth (I. bubalus). Many of the genetic and morphological identifications (ODWC) and Di-based (ODWC) assignments were in agreement. However, there are several instances where the morphological and Di-based assignments differed, and some instances where the genetic identity and morphologically and Di-based assignments disagreed. We have not definitively assigned any individuals to black buffalo (I. niger) using mitochondrial barcoding, however, two sequences were ambiguously assigned to this species. We will repeat the reactions for these two individuals to evaluate the assignment with additional data. We have a substantial number of samples for which barcoding data have not been generated. We are actively working*

on completing barcoding benchwork so that we may help refine species identities for the remaining samples. Since hybridization may be rampant among buffalofishes, mitochondrial DNA provides some insight into species identity, but due to its maternal inheritance may be discordant from the biparentally inherited autosomal genome. This phenomena is well documented in a variety of organisms, and discordance between mitochondrial genes and autosomal genes is commonly used to identify hybridized individuals in a population of interest (Wiens and Penkrot 2002, Pidancier et al. 2006, Leaché and Cole 2007, Hinojosa et al. 2019). In order to generate the autosomal genomic data we have elected to use genome-wide SNP data which we will generate through ddRAD library preps (Peterson et al. 2012). The ddRAD approach can generate many thousands of SNPs from throughout the genome for each individual which can then be used to infer population structure, species identity, hybridization, and effective population size. We have started the library preparation for generating the autosomal markers. In this library preparation process, DNA is digested with two restriction enzymes that perform double cuts which are thenindividually labelled with adapters and pooled for sequencing. We will generate ddRAD data for all of the samples that we currently have in hand. Our goal is to submit our first lane of sequencing by the end of April 2024, and to perform a second set of library preps and sequencing for any additional samples we receive by November 2024. In addition to the core of our work, the graduate student at OK State (K. Mapes) has been working to optimize a quantitative PCR based assay to measure the relative length of telomeres of each individual fish by comparing telomere copies to a single copy gene (in this case actin) to calculate a T/S ratio which provides an estimate of telomere length (Blackburn 1991, Cawthon 2002). We will evaluate telomere length estimated age in combination with otolith estimated age to determine if telomere lengths are reliable biomarkers of age in these three species of fish.

Overview 2025: Buffalofishes (Ictiobus cyprinellus, I. bubalus, I. niger) are three species of river- or lake-dwelling fish distributed in North America including Oklahoma that were previously believed to live for ~15 years, but recent discovery shows that I. cyprinellus individuals can live for over a century (Lackmann et al. 2019, Lackmann et al. 2021). Longevity of buffalofishes in Oklahoma is less extreme, however, I. bubalus has been shown to exceed 60 years (Snow, et al. 2020). Their longevity in combination with episodic recruitment suggests that population dynamics should be updated to ensure that effective management practices are in place. In Oklahoma, these fish are targets of recreational and tournament bowfishing, with large individuals that are likely older reproductive females being the prime targets (Scarnecchia and Schooley 2020). Furthermore, morphological identifications of buffalofish are confounded by hybridization (Stevenson 1964, Johnson and Minckley 1969), making morphological identification of species difficult (Schooley et al. 2023). Efforts to understand the aging and population dynamics of this fish will help us understand how to better manage and protect them if needed, as well provide us novel information on aging (Barnett et al. 2017, Love et al. 2019, Lackmann et al. 2021).

The primary objective of this grant is to use genetic methods to identify species and detect

hybridization. These insights complement morphologically determined identifications using traditional morphological traits and a depth index (Di = SL/BD) (Hubbs et al. 2008) for the three species of buffalofishes (Bart et al. 2010, Schooley et al. 2023). With these data, population age structures for each species of buffalofish will be calculated by ODWC biologists to assess the impacts of current management practices on these long-lived, episodically reproducing non-game fish.

Progress 2025 : The ODWC collected fin-clip samples from buffalofishes from 14 locations across Oklahoma (Figure 1) and stored in 100% ETOH prior to transport to OK State where they were kept refrigerated until extraction. During 2023 we optimized protocols for DNA extraction and refined and optimized PCR amplification for genetic barcoding using the cytochrome-b mitochondrial gene. In 2024 we completed DNA extraction for 960 individual fin samples and successfully barcoded a subset of these samples (n=388). Samples failed barcoding efforts for a variety of reasons, but often due to a failure to amplify or produce clean sequence. For each sample we used a qubit 4 fluorometer with the broad range assay to confirm that the DNA concentrations in the extracts were appropriate for the planned genetics work. The results from quantification confirmed that the DNA concentrations are more than sufficient for our goals of generating PCR-based barcoding and autosomal next generation sequencing.

The plans for the genetic data were to generate mitochondrial and autosomal markers for each sample and use these in combination to identify patterns of hybridization and introgression. During year 1 of the project, a protocol was successfully validated for the barcoding efforts for the cytochrome-b mitochondrial gene. The cytochrome-b mitochondrial gene (maternally inherited) is commonly used for genetic determination of species identity. Mitochondrial barcoding experiments were optimized by testing a series of primer combinations used in prior studies (Harris et al. 2002, Bagley et al. 2018) for three gene regions (cytochrome-b, COI, ND2), and have experimentally determined a set of PCR-amplification conditions that yield clean sequence reads for cytochrome-b using cytb514L and cytbH1591 primer combination with an annealing temperature of 50℃. Unfortunately, despite earlier validation of the PCR-protocol, extensive issues were encountered with PCR from May-August 2024. Several months were used to troubleshoot the repeated failure of PCR amplification. Troubleshooting entailed testing each reagent to ensure that they were without contamination, ordering new reagents and primers, diluting sample concentrations, testing several alternative PCR clean-up procedures, testing PCR-clean up modifications, and thermocycling protocols. One problem encountered was low yield PCRs that were insufficient for sequencing once the PCR product was cleaned in preparation for sequencing. Ultimately, the replacement of primers and a new PCR- master-mix formulation using modified ratios of key reagents resolved the issue. A new clean-up method was essential for successfully generating enough PCR amplicon quantity in sufficient concentration for sequencing. The troubleshooting experiments resulted in process delays

before commencing higher throughput PCR and sequencing of samples. Process improvements were culminated when samples were successfully PCR-amplified, confirmed that the amplified band is the expected size using gel electrophoresis, and ensured that the reactions are contaminant-free, and have completed sequencing. Each sample was bidirectionally sequenced to ensure that the basepair ambiguities were resolved and then aligned the sequences across using Sequencher Software (GeneCodes).

Genetic barcoding confirmed the species identity of 388 fish from nine waterbodies. The presence of all the three Ictiobus species that were previously posited to occur in Oklahoma based on morphological identifications. Genetic species ID was in agreement with visual morphology ID 62% of the time, while genetic ID was in agreement with morphologically-derived Di only 38% of the time. For the cytochrome-b gene we used BLAST (Camacho et al. 2009) searches through NCBI's Genbank portal to verify species identity. Previous collection efforts and its legal status as a Species of Greatest Conservation Need (due to inadequacies in population data) suggested that the Black Buffalo (I. niger) was present in Oklahoma reservoirs but potentially rare relative to Smallmouth or Bigmouth buffalofishes. This was supported by the genetic barcoding in that only 10.5% of individual samples barcoded were identified as Black Buffalo. In contrast, Smallmouth Buffalo and Bigmouth Buffalo comprised 67.4% and 22.1% of the samples, respectively.

Since hybridization may be rampant among buffalofishes, mitochondrial DNA provides some insight into species identity, but due to its maternal inheritance and the nature of mitochondrial introgression between species, it may be discordant from the biparentally inherited autosomal genome. This phenomenon is well documented in a variety of organisms, and discordance between mitochondrial genes and autosomal genes is commonly used to identify hybridized individuals (Wiens and Penkrot 2002, Pidancier et al. 2006, Leaché and Cole 2007, Hinojosa et al. 2019).

Genetic data were paired with age estimates from ODWC for a subsample of buffalofishes. In nine waterbodies (Figure 2), all three species of Ictiobus were collected, therefore these populations were included in an analyses to examine effects of species, locality, or the interaction effect of species and locality on age structure. A two-way ANOVA determined that locality had a significant effect on the response ($F=17.959$, $df=8$, $p<0.05$), whereas the species and the species-locality interaction effect were not significant ($p=0.153$ and $p=0.167$ respectively), indicating that spatial differences among reservoirs explain most of the observed variation in age structure. However, given that samples were collected using different methodologies over several years, other effects not examined (such as collecting gear biased toward certain sizes/ages) may have lurking significance.

2. Please describe and justify any changes in the implementation of your objective(s) or approach(es).

Our objectives have not changed from our proposed research plan to now. We additionally plan to continue optimization of a second mitochondrial barcoding gene (ND2 or COI) which may have increased variation relative to cytochrome-b and thus may provide additional resolution of species identities inferred from the maternal lineage. Additionally, we have added a new component to our work focused on telomere length and aging dynamics. We believe this additional research focus complements the goals of our ODWC partners in generating age structure data for each species within each sampled population.

3. If applicable, please share if the project resulted in any unexpected benefits, promising practices, new understandings, cost efficiencies, management recommendations, or lessons learned.

Many but not all of our genetic identifications are in agreement with morphological species identities and Di-based species identities for bigmouth and smallmouth buffalo. We are looking forward to having the genomic data in hand to establish which of these methods of species id can best serve as an accurate method of species identification moving forward, and also to assess if disagreement in species identities arises from hybridized individuals. If we are able to find that a particular identification method has high agreement with the genomic data then we anticipate that cost-effective identification practices can be established.

4. For Survey projects only: If applicable, does this project continue work from a previous grant? If so, how do the current results compare to prior results? (Recipients may elect to add attachments such as tables, figures, or graphs to provide further detail when answering this question.)
Not Applicable

5. If applicable, identify and attach selected publications, photographs, screenshots of websites, or other documentation (including articles in popular literature, scientific literature, or other public information products) that have resulted from this project that highlight the accomplishments of the project.

While under the 2023 buffalofishes grant, a vital step in the process was the communication of this science to both the scientific community and the public. Hypothesis and initial data were communicated by Mapes through the 3MT, 3 Minute Thesis, competition in which she won first place for Oklahoma State University's College of Arts and Sciences. In this competition, Mapes had three minutes to explain the complex aging dynamics of buffalofishes and our research efforts, in a way that was interesting and digestible to the non-scientific community using a single slide (attached). Mapes moved forward into the university-wide competition presenting to a large general public audience.

6. Is this a project you wish to highlight for communication purposes?
No

Questionnaire Attachments

Note: Some attachments listed here may not appear in the Appendix due to file incompatibility. All attachments can be accessed using the links below.

Descriptive Name	Field Tags	Attachment Type
F22AF02999 (T-129-R-1)	• Objective Completion Progress	Performance Report / Performance Hard Copy Report

Appendix

Genetic Identification and Estimation of Population Demographics for Oklahoma Buffalofish (*Ictiobus bubalus*, *I. cyprinellus, I. niger)*
F22AF02999 (T-129-R-1)

Project Duration: January 1, 2023 – June 30, 2025
Final Report: January 26, 2026

Principal Investigator:
Dr. Guinevere O.U. Wogan.
501 Life Sciences West, Department of Integrative Biology
Oklahoma State University
Stillwater OK 74078
415-336-9216
gwogan@okstate.edu

Objective 1: Complete 1 investigation by June 30, 2025

TRACS Questionnaire

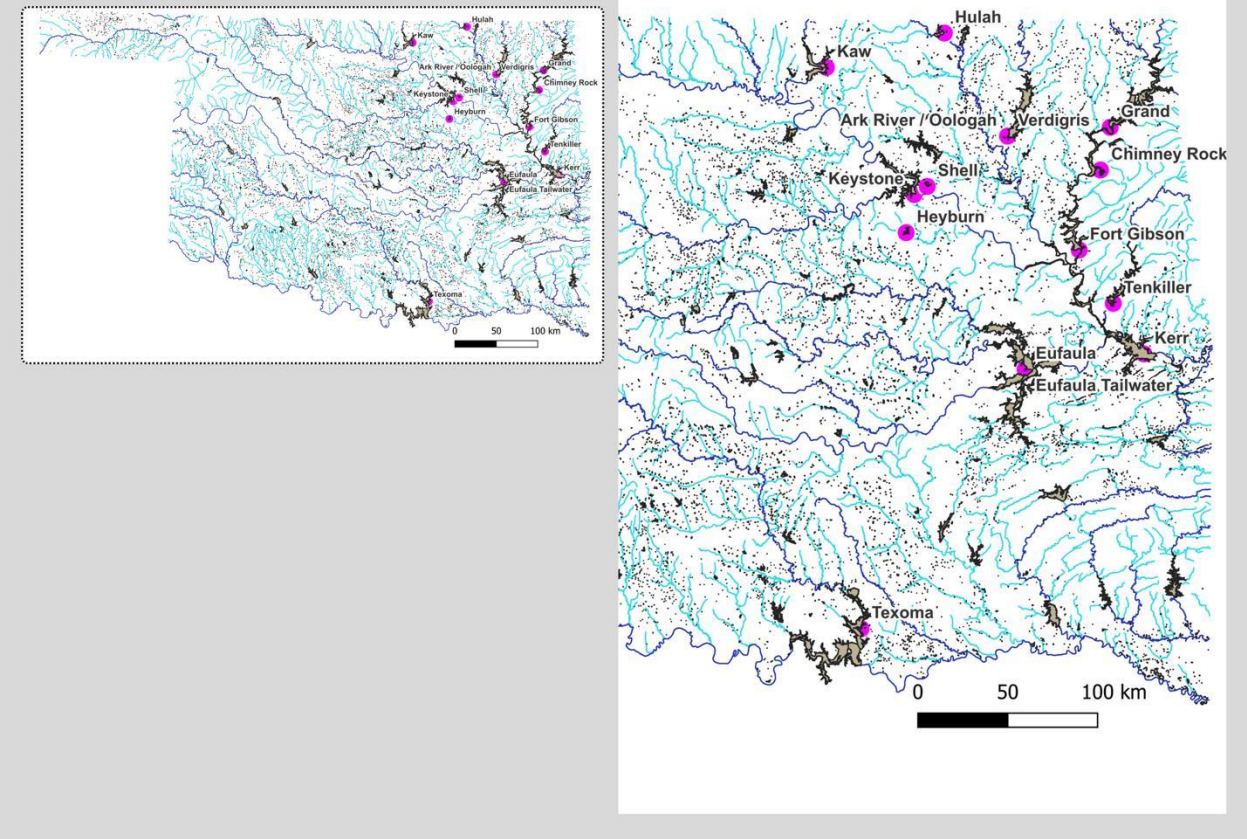
Q1. What progress has been made towards completing the objectives of the project?
Overview: Buffalofishes (*Ictiobus cyprinellus, I. bubalus, I. niger*) are three species of river- or lake-dwelling fish distributed in North America including Oklahoma that were previously believed to live for ~15 years, but recent discovery shows that I. cyprinellus individuals can live for over a century (Lackmann et al. 2019, Lackmann et al. 2021). Longevity of buffalofishes in Oklahoma is less extreme, however, I. bubalus has been shown to exceed 60 years (Snow, et al. 2020). Their longevity in combination with episodic recruitment suggests that population dynamics should be updated to ensure that effective management practices are in place. In Oklahoma, these fish are targets of recreational and tournament bowfishing, with large individuals that are likely older reproductive females being the prime targets (Scarnecchia and Schooley 2020). Furthermore, morphological identifications of buffalofish are confounded by hybridization (Stevenson 1964, Johnson and Minckley 1969), making morphological identification of species difficult (Schooley et al. 2023). Efforts to understand the aging and population dynamics of this fish will help us understand how to better manage and protect them if needed, as well provide us novel information on aging (Barnett et al. 2017, Love et al. 2019, Lackmann et al. 2021).

The primary objective of this grant is to use genetic methods to identify species and detect hybridization. These insights complement morphologically determined identifications using traditional morphological traits and a depth index (Di = SL/BD) (Hubbs et al. 2008) for the three species of buffalofishes (Bart et al. 2010, Schooley et al. 2023). With these data, population age structures for each species of buffalofish will be calculated by ODWC biologists to assess the impacts of current management practices on these long-lived, episodically reproducing non-game fish.

Progress: The ODWC collected fin-clip samples from buffalofishes from 14 locations across Oklahoma (Figure 1) and stored in 100% ETOH prior to transport to OK State where they were kept refrigerated until extraction. During 2023 we optimized protocols for DNA extraction and refined and optimized PCR amplification for genetic barcoding using the cytochrome-b mitochondrial gene. In 2024 we completed DNA extraction for 960 individual fin samples and successfully barcoded a subset of these samples (n=388). Samples failed barcoding efforts for a variety of reasons, but often due to a failure to amplify or produce clean sequence.

For each sample we used a qubit 4 fluorometer with the broad range assay to confirm that the DNA concentrations in the extracts were appropriate for the planned genetics work. The results from quantification confirmed that the DNA concentrations are more than sufficient for our goals of generating PCR-based barcoding and autosomal next generation sequencing.

Figure 1. Map of Oklahoma with 14 source populations of genetic samples for buffalofishes (*Ictiobus* spp.) noted by magenta dots.



The plans for the genetic data were to generate mitochondrial and autosomal markers for each sample and use these in combination to identify patterns of hybridization and introgression. During year 1 of the project, a protocol was successfully validated for the barcoding efforts for the cytochrome-b mitochondrial gene. The cytochrome-b mitochondrial gene (maternally inherited) is commonly used for genetic determination of species identity. Mitochondrial barcoding experiments were optimized by testing a series of primer combinations used in prior studies (Harris et al. 2002, Bagley et al. 2018) for three gene regions (cytochrome-b, COI, ND2), and have experimentally determined a set of PCR-amplification conditions that yield clean sequence reads for cytochrome-b using cytb514L and cytbH1591 primer combination with an annealing temperature of 50°C. Unfortunately, despite earlier validation of the PCR-protocol, extensive issues were encountered with PCR from May-August 2024. Several months were used to troubleshoot the repeated failure of PCR amplification. Troubleshooting entailed testing each reagent to ensure that they were without contamination, ordering new reagents and primers, diluting sample concentrations, testing several alternative PCR clean-up procedures, testing PCR-clean up modifications, and thermocycling protocols. One problem encountered was low yield PCRs that were insufficient for sequencing once the PCR product was cleaned in preparation for sequencing. Ultimately, the replacement of primers

and a new PCR- master-mix formulation using modified ratios of key reagents resolved the issue. A new clean-up method was essential for successfully generating enough PCR amplicon quantity in sufficient concentration for sequencing. The troubleshooting experiments resulted in process delays before commencing higher throughput PCR and sequencing of samples. Process improvements were culminated when samples were successfully PCR-amplified, confirmed that the amplified band is the expected size using gel electrophoresis, and ensured that the reactions are contaminant-free, and have completed sequencing. Each sample was bidirectionally sequenced to ensure that the basepair ambiguities were resolved and then aligned the sequences across using Sequencher Software (GeneCodes).

Genetic barcoding confirmed the species identity of 388 fish from nine waterbodies. The presence of all the three *Ictiobus* species that were previously posited to occur in Oklahoma based on morphological identifications. Genetic species ID was in agreement with visual morphology ID 62% of the time, while genetic ID was in agreement with morphologically-derived Di only 38% of the time. For the cytochrome-b gene we used BLAST (Camacho et al. 2009) searches through NCBI's Genbank portal to verify species identity. Previous collection efforts and its legal status as a Species of Greatest Conservation Need (due to inadequacies in population data) suggested that the Black Buffalo (*I. niger*) was present in Oklahoma reservoirs but potentially rare relative to Smallmouth or Bigmouth buffalofishes. This was supported by the genetic barcoding in that only 10.5% of individual samples barcoded were identified as Black Buffalo. In contrast, Smallmouth Buffalo and Bigmouth Buffalo comprised 67.4% and 22.1% of the samples, respectively.

Since hybridization may be rampant among buffalofishes, mitochondrial DNA provides some insight into species identity, but due to its maternal inheritance and the nature of mitochondrial introgression between species, it may be discordant from the biparentally inherited autosomal genome. This phenomenon is well documented in a variety of organisms, and discordance between mitochondrial genes and autosomal genes is commonly used to identify hybridized individuals (Wiens and Penkrot 2002, Pidancier et al. 2006, Leaché and Cole 2007, Hinojosa et al. 2019).

Genetic data were paired with age estimates from ODWC for a subsample of buffalofishes. In nine waterbodies (Figure 2), all three species of *Ictiobus* were collected, therefore these populations were included in an analyses to examine effects of species, locality, or the interaction effect of species and locality on age structure. A two-way ANOVA determined that locality had a significant effect on the response ($F=17.959$, $df=8$, $p<0.05$), whereas the species and the species-locality interaction effect were not significant ($p=0.153$ and $p=0.167$ respectively), indicating that spatial differences among reservoirs explain most of the observed variation in age structure. However, given that samples were collected using different methodologies over several years, other effects not examined (such as collecting gear biased toward certain sizes/ages) may have lurking significance.

Figure 2. Summary statistics for estimated age of buffalofishes collected from nine populations in Oklahoma.

Species	Locality	Mean Age	Median Age	SD Age	Min Age	Max Age	n
bubalus	Chimney Rock	32.75	32	9.996427933	19	51	8
bubalus	Fort Gibson	9.25	8	7.391240409	1	33	24
bubalus	Grand	20.8596491	21	9.375954421	3	41	57
bubalus	Hulah	15.6	13	9.233479456	4	39	15
bubalus	Keystone	16.5	14.5	5.802298395	12	25	4
bubalus	Shell	27.6909091	26	13.34148741	8	61	55
bubalus	Tenkiller	22.3478261	20	11.75356308	6	55	23
bubalus	Texoma	14.8235294	12	7.939106484	4	31	17
bubalus	Verdigris	8.8	5	11.54123044	1	29	5
cyprinellus	Chimney Rock	41	41	NA	41	41	1
cyprinellus	Fort Gibson	5	5	2.943920289	2	8	4
cyprinellus	Grand	22.0909091	22	11.44075649	4	40	11
cyprinellus	Hulah	12.6086957	11	5.750408131	4	28	23
cyprinellus	Keystone	21.25	20	8.057087977	13	32	4
cyprinellus	Shell	41.3333333	49	19.66596044	12	69	9
cyprinellus	Tenkiller	26.75	24	14.97497913	14	45	4
cyprinellus	Texoma	15.6	19.5	8.871928263	1	25	10
cyprinellus	Verdigris	8.25	6.5	7.410578025	2	18	4
niger	Chimney Rock	23	23	NA	23	23	1
niger	Fort Gibson	4.66666667	6	3.214550254	1	7	3
niger	Grand	29	29	1.414213562	28	30	2
niger	Hulah	13	13	5.206833117	4	23	10
niger	Keystone	14	14	4.242640687	11	17	2
niger	Shell	15	15	2.828427125	13	17	2
niger	Tenkiller	27.5	27	14.11027994	8	47	6
niger	Texoma	19.3333333	17	16.62327685	4	37	3
niger	Verdigris	3	3	NA	3	3	1

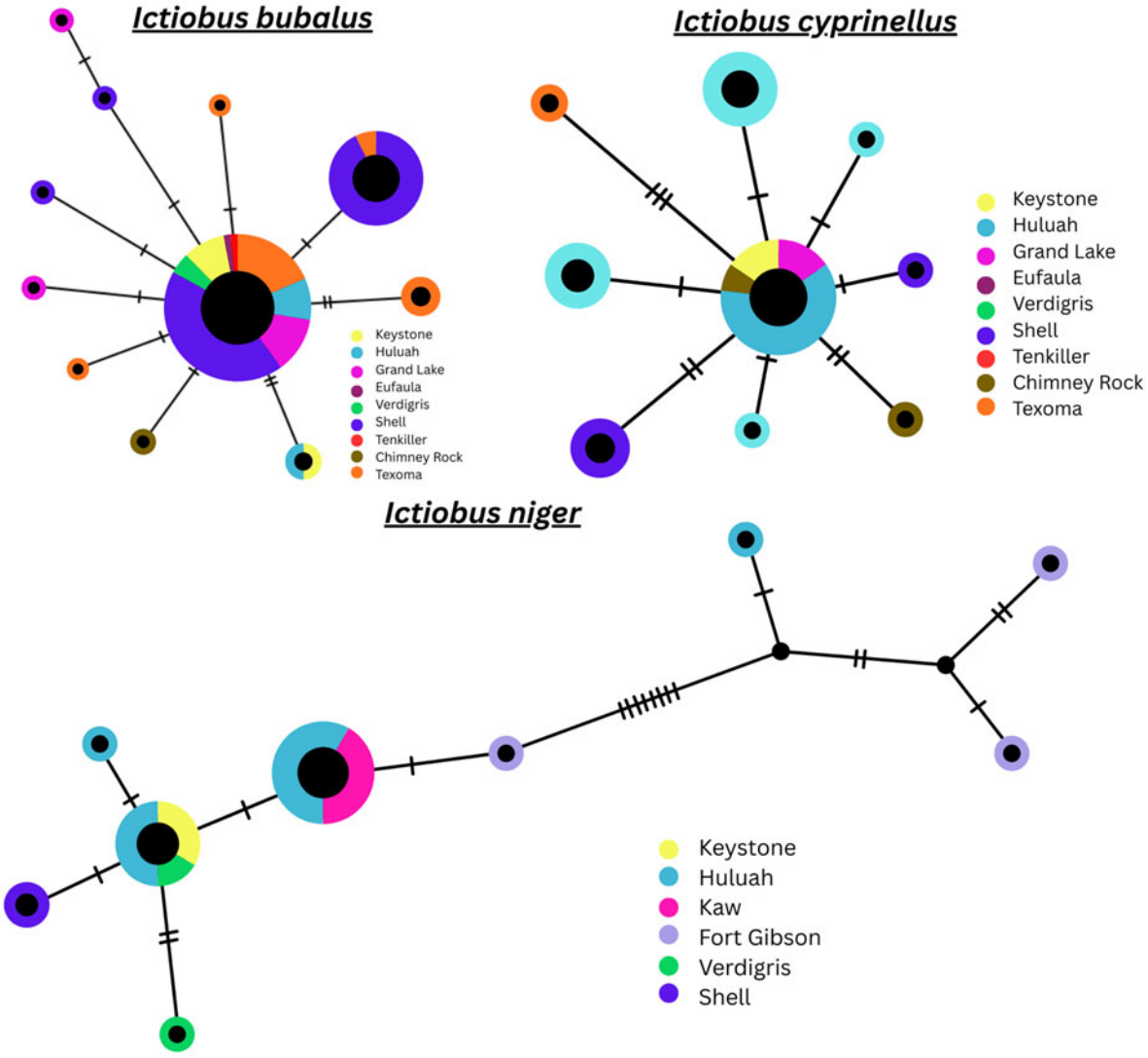
Figure 3. Results of two-way ANOVA examining effects of species and location on age structure for buffalofishes in Oklahoma.

	Df	Sum Sq	Mean Sq	F Value	Pr (>F)
Species	2	419	209.3	1.891	0.153
Locality	8	15903	1987.9	17.959	<2e-16***
Species : Locality	16	2389	149.3	1.349	0.167
Residuals	281	31104	110.7		
Signif. Codes	0 '***'	0.001 '**'	0.01 '*'	0.05 '.'	

A haplotype network was developed from SNP results for Oklahoma buffalofishes considering the populations sampled in this project (Figure 4). Within the haplotype network, each circle represents a unique mitochondrial haplotype, with circle area proportional to the

number of individuals sharing that haplotype and colors indicating sampling localities. Hash marks on connecting branches indicate single mutational steps between haplotypes, and small black circles represent unsampled or inferred intermediate haplotypes. Separate networks are shown for *I. bubalus*, *I. cyprinellus*, and *I. niger* to illustrate within-species genetic diversity and geographic structuring of haplotypes. The starburst networks of *I. bubalus* and *I. cyprinellus* suggest that there was a widespread ancestral haplotype and a rapid demographic expansion likely that occurred as fish colonized different river or stream habitats. Subsequent construction of reservoir dams likely prevented historical patterns of connectivity resulting in the segregation and maintenance of different haplotype combinations in distinct populations. However, hybridization among the three species within reservoirs may obscure or complicate some of these within-population/species haplotypes.

Figure 4. Haplotype networks for three Ictiobus species from Oklahoma waterbodies.



In consideration of the mitochondrial barcoding, spatial distribution of buffalofish stocks, population age structure, and haplotype networks, project results indicate several key conclusions: First- Oklahoma buffalofish populations are dominated by Smallmouth Buffalo, with

fewer bigmouth and black buffalofishes. Second- species composition varies among waterbodies rather than being uniform statewide. Third- locality, rather than species identity, emerges as a primary statistical effect on age variation (rather than species), with some waterbodies supporting older, demographically skewed populations, while others contain younger, more homogenous age structures. And fourth- haplotype networks reveal multiple shared and locality-specific haplotypes, consistent with limited but not entirely absent connectivity among populations in this fragmented freshwater landscape. Together, these results establish a geographically structured mosaic of species composition, demographic profiles, and genetic variation that provides essential context for subsequent work on telomere dynamics and gene expression in long-lived buffalofishes. These results, paired with detailed assessments of population dynamics, morphological characteristics, age structure, and observed recruitment patterns allow for a more thorough examination the status and distribution of Oklahoma buffalofishes in eastern Oklahoma for their sustainable management within emerging and evolving harvest fisheries. Further, these results provide key insights into the development of regulatory and conservation strategies for Black Buffalo, as it exists within an admixture of morphologically and genetically similar species within the same genus.

In addition to the core objective, this work presented opportunities to preliminarily examine related hypotheses on the relationship between telomere length and age for Oklahoma buffalofishes. Some samples were optimized with a quantitative PCR based assay to measure the relative length of telomeres of each individual fish by comparing telomere copies to a single copy gene (in this case, actin) to calculate a T/S ratio which provides an estimate of telomere length (Blackburn 1991, Cawthon 2002). The genetic assay to determine telomere length was used in combination with otolith estimated age to determine if telomere lengths are reliable biomarkers of age in these three species of fish in Oklahoma. These efforts are instrumental in developing a specialized plasmid to standardize the calculation of the T/S ratio across qPCR runs/ batches. These efforts may be considered for future studies.

Q2. Please describe and justify any changes in the implementation of your objectives(s) or approach(es)

The primary objectives did not change from the proposed research plan: to use genetics methods to confirm the species identity of buffalofish and detect hybridization and population dynamics among species in Oklahoma freshwater lakes. The number of samples available for this work was increased, requiring that each major objective required more time to complete. Therefore, the main change was the revised the timeline for completing each task. The timeline for the work was extended by one year. Previously, an additional component focused on telomere length and aging dynamics was added to the genetic assay for this work. This additional research focus complements the original goals in generating age-structure data for each species within sampled populations and as a complement to the otolith-based age calculations.

References

Bagley, J.C., Mayden, R.L. & Harris, P.M. (2018) Phylogeny and divergence times of suckers (cypriniformes: Catostomidae) inferred from bayesian total-evidence analyses of molecules, morphology, and fossils. PeerJ, 6, e5168. https://doi.org/10.7717/peerj.5168

Barnett, L.A.K., Branch, T.A., Ranasinghe, R.A. & Essington, T.E. (2017) Old-growth fishes become scarce under fishing. Current Biology, 27, 2843-2848.e2842. https://doi.org/10.1016/j.cub.2017.07.069

Bart, H.L., Clements, M.D., Blanton, R.E., Piller, K.R. & Hurley, D.L. (2010) Discordant molecular and morphological evolution in buffalofishes (actinopterygii: Catostomidae). Molecular Phylogenetics and Evolution, 56, 808-820. https://doi.org/10.1016/j.ympev.2010.04.029

Blackburn, E.H. (1991) Structure and function of telomeres. Nature, 350, 569-573. 10.1111/ele.13426

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. & Madden, T.L. (2009) Blast+: Architecture and applications. BMC Bioinformatics, 10, 421

Cawthon, R.M. (2002) Telomere measurment by quantitative pcr. Nucleic Acids Research, 30, e47. 10.1093/nar/30.10.e47

Harris, P.M., Mayden, R.L., Perez, H.E. & Garcia de Leon, F. (2002) Phylogenetic relationships of redhorse (moxostoma) and jumprock (scartomyzon) suckers (cypriniformes:Catostomidae) based on mitochondrial cytochrome b sequence data. Journal of Fish Biology, 61, 1433-1452

Hinojosa, J.C., Koubínová, D., Szenteczki, M.A., Pitteloud, C., Dincă, V., Alvarez, N. & Vila, R. (2019) A mirage of cryptic species: Genomics uncover striking mitonuclear discordance in the butterfly thymelicus sylvestris. Molecular Ecology, 28, 3857-3868. https://doi.org/10.1111/mec.15153

Hubbs, C., Edwards, R.J. & Garrett, G.P. (2008) An annotated checklist of the freshwater fishes of texas, with keys to identification of species. Texas Academy of Science

Johnson, D.W. & Minckley, W. (1969) Natural hybridization in buffalofishes, genus ictiobus. Copeia, 1969, 198-200

Lackmann, A.R., Andrews, A.H., Butler, M.G., Bielak-Lackmann, E.S. & Clark, M.E. (2019) Bigmouth buffalo ictiobus cyprinellus sets freshwater teleost record as improved age analysis reveals centenarian longevity. communications biology, 2. 10.1038/s42003-019-0452-0

Lackmann, A.R., Kratz, B.J., Bielak-Lackmann, w.S., Jacobson, R.I., Sauer, D.J., Andrews, A.H., Butler, M.G. & Clark, M.E. (2021) Long-lived population demographics in a declining, vulnerablefishery—bigmouth buffalo (ictiobus cyprinellus) of jamestownreservoir, north dakota. Canadian Journal of Fisheries and Aquatic Sciences, 78, 1486-1496. 10.1139/cjfas-2021-0485

Leaché, A. & Cole, C. (2007) Hybridization between multiple fence lizard lineages in an ecotone: Locally discordant variation in mitochondrial DNA, chromosomes, and morphology. Mol. Ecol., 16, 1035-1054. 10.1111/j.1365-294X.2006.03194.x

Love, S.A., Tripp, S.J. & Phelps, Q.E. (2019) Age and growth of middle mississippi river smallmouth buffalo. The American Midland Naturalist, 182, 118-123, 116

Pidancier, N., Jordan, S., Luikart, G. & Taberlet, P. (2006) Evolutionary history of the genus capra (mammalia, artiodactyla): Discordance between mitochondrial DNA and y-chromosome phylogenies. Mol. Phylogenet. Evol., 40, 739-749. 10.1016/j.ympev.2006.04.002

Scarnecchia , D.L. & Schooley, J.D. (2020) Bowfishing in the united states: History, status, ecological impact, and a need for management. Transactions of the Kansas Academy of Science, 123, 285-338, 254

Schooley, J.D., Gainer, C., Taylor, C. & Pallett, M. (2023). Oklahoma nongame fishes research and management. Oklahoma Department of Wildlife Conservation

Stevenson, J. (1964) Fish farming experiment station. US Fish and Wildlife Service Circular, 179, 79-100

Wiens, J.J. & Penkrot, T.A. (2002) Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (sceloporus). Syst. Biol., 51, 69-91. 10.1080/106351502753475880