Project Title: Abundance and distribution of early life stages of invasive carp in Red River Basin.

Geographic Location: Red River Basin

Lead Agency: Louisiana Department of Wildlife and Fisheries (LDWF), Robert P. Bourgeois (rbourgeois@wlf.la.gov)

Participating Agencies: Louisiana Department of Wildlife and Fisheries (LDWF) Nicholls State University (Quenton Fontenot, NSU); U.S. Fish and Wildlife Service (Oklahoma Fish and Wildlife Conservation Office)

Statement of Need:
Successful management of invasive carp is dependent on a thorough understanding of life histories and interactions with local environments. Reproduction, a key component of life history, can be used to guide control efforts by identifying where large spawning aggregations occur, and by identifying source and sink populations.

The purpose project is to determine where successful reproduction of invasive carp is occurring through the identification and cataloging of larval fish samples in Louisiana and Oklahoma waters. The documentation of the presence/absence of invasive carp larvae will assist in determining the leading edge of expansion throughout the Red River Basin and will help document where self-sustaining populations have established. This project will inform the direct management activities to contain the spread of invasive carp such as placement of deterrents and possible locations for other control activities.

In Louisiana, the Red, Atchafalaya, and Mississippi Rivers are connected at the Old River complex, which has allowed invasive carp to spread to all three rivers, and further spread to smaller rivers in LA through natural and man-made connections such as flood relief or freshwater diversions used for wetland restoration. Commercial navigation channels have allowed migration between river basins, thereby allowing invasive carp and other fish to move

freely between basins. The LDWF began monitoring ichthyoplankton in 2013 to better understand the extent of invasive carp reproduction and to quantify larval invasive carp in Louisiana. In 2013, the samples were taken in April, May, and June. This project was modified to have the samples taken in May, June, and July in 2014 to try to define a temporal pattern of the presence of invasive carp early life stages. Sample sites were revisited in 2019 to see if invasive carp reproduction has remained constant over time or has spread to other areas where carp reproduction was not detected in 2013-14. The 2019 samples were taken during a historic flood on the Mississippi and Red Rivers and the data does not reflect what we would expect of a "typical" year's reproduction. Future studies will either confirm changes in reproductive patterns as reflected by the 2019 results or that the 2013 and 2014 years are more typical.

The long-term goal for the research conducted through this early life stage study, in conjunction with telemetry-based movement studies, is to determine if it would be possible to direct harvest or place barriers or deterrents to further restrict invasive carp movement and reproduction.

In Oklahoma, efforts to evaluate recruitment by invasive carp species in the Red River have not yet been conducted. The OKFWCO began monitoring for the presence of larval Bighead Carp (*Hypophthalmichthys nobilis*), Silver Carp (*Hypophthalmichthys molitrix*), Grass Carp (*Ctenopharyngodon idella*), and Black Carp (*Mylopharyngodon piceus*) in 2021 to determine the distribution and extent of successful reproduction by these species in the Red River and its tributaries. Initiating these assessments for understudied systems will inform long-term control strategies and provide a baseline for monitoring the extent of invasion in Oklahoma and surrounding states.

The Red River flows east in Oklahoma to form its border with Texas. This section of the Red River is characterized by numerous southern-flowing tributaries, which typically possess an impoundment and an associated reservoir above. While these impoundments are considered to be impassable by invasive carp species, the connectivity of the Red River with the lower reaches of its tributaries foster the appropriate conditions for spawning below the dams, as well as the potential for bait fish transfer to nearby reservoirs. This region supports a thriving recreational fishery that could potentially be impacted by invasive carp invasion.

This study supports the objectives of the Lower Mississippi River Basin invasive carp control plan, by helping to determine what aquatic habitats in Louisiana and Oklahoma are suitable for invasive carp spawning. When combined with other studies it may help determine what populations are source populations and help in directing efforts for harvest or exclusion.

Project Objectives:

1. Determine the extent of invasive carp spawning activity in the Red River basin.

Project Highlights:

- Invasive carp are reproducing in the majority of the Red River sites sampled in LA.
- Invasive carp comprise 14% of the ichthyoplankton samples collected in the Red River, LA in 2022.
- Invasive carp spawned primarily in May and June in LA.
- Twenty-one samples ichthyoplankton samples from Oklahoma (out of 62 samples) had been processed so no conclusions can be made until all sample processing and analysis is completed. Light trap samples and qPCR samples are still being processed.

Methods:

Louisiana:

The samples collected in 2021 informed the locations of this year of the study. Ichthyoplankton samples were collected at 14 sites throughout Louisiana by LDWF personnel in April, May, and June of 2022. At each sample station, Ichthyoplankton samples were collected by towing a 0.5m diameter 500µm mesh ichthyoplankton net just below the water surface for a duration of 10 minutes per tow. A separate tow was made on the left, middle, and right portions of the channel. Samples were preserved in 70% ethanol or isopropyl alcohol and delivered to NSU.

Upon arrival at NSU, larval and juvenile fish were separated from debris for each sample and stored in plastic containers or scintillating vials containing 70% ethanol or isopropyl alcohol. Each fish was identified to at least the family taxonomic level. Cyprinids were further identified as either an invasive carp species or not an invasive carp species. All fish were identified fishes to family based on Auer (1982). Identification of cyprinids such as invasive carp larvae were based on Chapman (2006), Chapman and George (2011) and George and Chapman (2013). Taxonomic classification beyond family for non-cyprinids was based on Auer (1982).

Oklahoma:

Ichthyoplankton Tows.— During May–August, ichthyoplankton tows were conducted using nets homologous to those used by LDWF, also in triplicates across the river channel as described previously. Nets were outfitted with a mechanical flowmeter (General Oceanics, Inc.; Model 2030R) mounted across the mouth to quantify the volume of water sampled (m^3). Staff deployed tow nets in tandem with one sampling the upper water column and the other sampling mid-column. Due to excessive debris loading in the past, the target duration for tows was three minutes each. Samples were preserved in 95% nondenatured ethanol. To reduce DNA cross-contamination, all gear used during sampling will be soaked in either 2% Virkon solution for 20 minutes or 20% bleach solution for at least 10 seconds before hanging in the sunlight to be dried.

Light Trapping.—During the ichthyoplankton sampling season, we concurrently deployed Quadrafoil Light Traps (WaterMark, Jackson, MS) in the Red River and its tributaries between the Denison Dam and the Arkansas state line (Fig.2). These cylindrical traps are characterized by their series of polycarbonate tubes arranged in a cloverleaf shape and lower a collection chamber where contents are funneled. In the center of the tubes, a green LED light (FishXtrada/Lumica USA, Inc., Anaheim, CA) was suspended to attract phototactic aquatic organisms, such as macroinvertebrates, and numerous species of juvenile fishes, including invasive carp (Roth 2018; Brandenburg et al. 2019). Traps were anchored in place and floated with an agency-labeled buoy attached. The majority of our light trapping was done in eddies, side channels, floodplains, and small tributaries (especially during dam discharge events), which often serve as habitats for drifting larvae to reside and develop into free-swimming mesolarvae. Tandem sets of floating and sinking traps sampled the upper and lower water columns, respectively. Traps were anchored

in place with an agency-labeled buoy attached and retrieved after approximately 24 hours. Sample preservation and equipment sanitation protocols for light trapping were as described for those of ichyhyoplankton tows.

Sample Preparation.—Each sample's ethanol solution was replaced within 48 hours of collection to remove DNA not directly associated with its specimens and reduce dilution for better preservation (Kelso et al. 2012; Nagy 2010; U.S. Fish and Wildlife Service n.d.). This consisted of pouring contents of each sample through a sanitized 500-µm stainless steel sieve, placing contents back into their bottle, and adding fresh 95% ethanol solution. Dense/debris-heavy samples had ethanol replaced two or three times. Samples were then gently agitated and left to rest until contents have settled. At this point, we used a sanitized 2-mL pipette to extract three aliquots of ethanol solution and deposit 1-mL into leak-proof microcentrifuge tubes.

Due to the time consuming-nature of sorting and identifying larval fish, the aliquots were sent to the Whitney Genetics Lab (USFWS) in December 2022 for subsequent DNA screening. This tool will be used to identify Silver and Bighead Carp DNA concentrations in each sample. Using the results as a guide, samples will then be prioritized for visual identification based on their quantity of target DNA.

DNA Extraction.—The samples which have been collected and subsampled in triplicate/location as outlined above were stored in 1.5 mL centrifuge tubes for DNA extraction at Whitney Genetics Lab. While extracting, each of the three tubes that constitute one sample will be kept separate. Samples will be centrifuged at max speed (~18,000 rpms) to concentrate any cells and/or eDNA at the very bottom of the MCT (microcentrifuge tube), the supernatant will then be discarded, and the samples will be set to dry in a 60°C bead bath for 15 minutes to remove any excess ethanol. Ethanol is a well-documented PCR inhibitor, so care must be taken to ensure each sample is dry before continuing with subsequent steps. Once the samples in their individual MCTs are dry, they will be frozen in -20°C storage to preserve the eDNA until ready to extract. Since there's no need for a swab in this setting, (because the sample will be dried in a 1.5ML tube already), DNA will be extracted using a slightly modified version of the

commercially available gMax mini genomic DNA extraction kit (IBI scientific) and final extracts will have a final volume of 200µL elution buffer.

qPCR.—DNA extracts will be analyzed using quantitative PCR (qPCR) to determine the presence of Silver or Bighead carp DNA. We will use a multiplex qPCR assay that contains six total loci. The qPCR multiplex includes two general invasive carp markers (i.e., can detect Silver and Bighead carp, but cannot differentiate between the two species) located on the cytochrome oxidase I gene of both species of carp (ACTM 1/3), two markers specific to Silver Carp located on the ND6 and ND2 regions of the mitochondria (SCTM 4/5), and two markers specific to Bighead Carp located on the ND4 and ND6 regions of the mitochondria (BHTM1/2) (Farrington et. al 2016). qPCR master mix concentrations and thermal profiles will follow standard USFWS lab protocols for these markers developed for eDNA monitoring (USFWS 2022). All qPCR will be conducted on a liquid handling robot (Eppendorf EP motion 5075) and each sample will be analyzed in replicates of eight. Any sample that has at least one detection for any of the eight replicates will be considered a positive detection. It is important to point out that a positive detection does not indicate that carp eggs or larvae are present in the original sample. DNA from carp can collect on sampling gears and could be collected from the water column during field sampling and previous use of these methods have demonstrated that eggs and larvae are not always present in samples that have positive qPCR detections (Fritts et al. 2019)

Sorting and identification.— From December 2022 to approximately April 2023, while qPCR analyses are run, the OKFWCO will be manually removing debris from samples to isolate specimens for identification. As qPCR results become available, samples will be prioritized for visual identification based on their concentrations of Bighead and Silver Carp DNA markers. Fish will be identified to family based on characteristics described by Auer (1982). Narrowed identification of Cyprinids to genus will completed using Holland-Bartels et al. (1990), Taber (1969), May and Gasaway (1967), and Snyder (1979). Potential invasive carp genera were identified using Chapman (2006), Chapman and George (2011) and George and Chapman (2013). We will examine fishes with two stereo microscopes: an Amscope SM-4TPZZ (3.5x–180x total magnification) and a Nikon SMZ800N stereo microscope (5x–480x total magnification).

Genetic Confirmation.—Larval fish and eggs often suffer from deformation, loss of pigmentation, and significant shrinkage, particularly in highly concentrated alcohol fixatives and towed nets (Kelso and Rutherford, 1996; Smith and Walker, 2003; Frimpong and Henebry 2012; König and Borcherding, 2012; Larson et al. 2016). Bigheaded Carp are also difficult to distinguish accurately (Yi et al. 1988; Chapman 2006; Chapman and George 2011; George and Chapman 2013, 2015). Therefore, individual eggs and larvae identified as potential invasive carp, or specimens deemed unidentifiable, will be separated for genetic analysis by the U.S. Fish and Wildlife Service's Southwest Native Aquatic Resource and Recovery Center (SNARRC) in Dexter, New Mexico to confirm species identity.

Results and Discussion:

Louisiana:

Table 1 lists the results of the 99 samples collected in Louisiana. Samples are broken down by family composition with invasive carp making up 1,975 of the 14,007 larvae collected (14.01%). Clupeidae was most abundant and occurred in 88 samples. Ictaluridae was the least abundant and occurred in only two samples. Invasive carp were the second most abundant species represented. There are also unidentified yolk-sac larvae (N=2) and some larvae (N=103) that were too degraded to confirm identification.

Invasive carp were identified in samples from April, May and June (Table 2) with eight out of 11 site samples having invasive carp larvae present (Figure 1). Only two invasive carp were present in April. These low sample numbers may suggest that reproduction may commence in April but doesn't ramp up until later in the spring, Samples collected in May included 981 invasive carp larvae while those collected in June included 992 invasive carp larvae. The largest single site total of 659 invasive carp larvae was collected in May. In 2022, invasive carp have increased both in numbers and in spatially compared to previous sampling in the Red River of LA. Sample site location information is listed in Appendix 1

Table 1. Total number of larvae identified within each family. Frequency represents the number of samples that contained at least one individual in that family. Total number and frequency of larvae that could not be identified due to degradation or at the yolk-sac stage also listed. Invasive carp were the second most abundant larvae and occurred in 37 samples. Four samples had no larvae present.

Family	Frequency	Number
Clupeidae	88	9,896
Invasive Carp	37	1,975
Catostomidae	71	902
Centrarchidae	48	480
Cyprinidae	39	323
Sciaenidae	22	174
Percichthyidae	17	78
Atherinidae	23	39
Lepisosteidae	6	14
Percidae	11	11
Poeciliidae	6	8
Ictaluridae	2	2
Subtotal		**13,902**
Unidentified Degraded	27	103
Unidentified Yolk-sac Larvae	2	2
Subtotal		**105**
Total Fish		**14,007**

Table 2. Total number of invasive carp larvae collected by month and sample station. The frequency indicates the number of net tows that collected invasive carp from that station. For example, if the frequency is one, then only one net tow at that station collected invasive carp.

Month	River	Station	Frequency	Number
April	Red	.	1	2
May	Red	.	3	659
May	Red	Hwy 2	2	45
May	Red	I-220	3	167
May	Red	Pool 3 – Hamptons 3	1	1
May	Red	South	3	109
			Subtotal	**981**
June	Red	.	1	7
June	Red	Hwy 2	3	108
June	Red	I-220	3	210
June	Red	Pool 3 – Grand Ecore 1	2	12
June	Red	Pool 3 – Grand Ecore 2	3	256
June	Red	Pool 3 – Hamptons 3	2	34
June	Red	Pool 4 – Coushatta 1	3	45
June	Red	South	3	320
			Subtotal	**992**

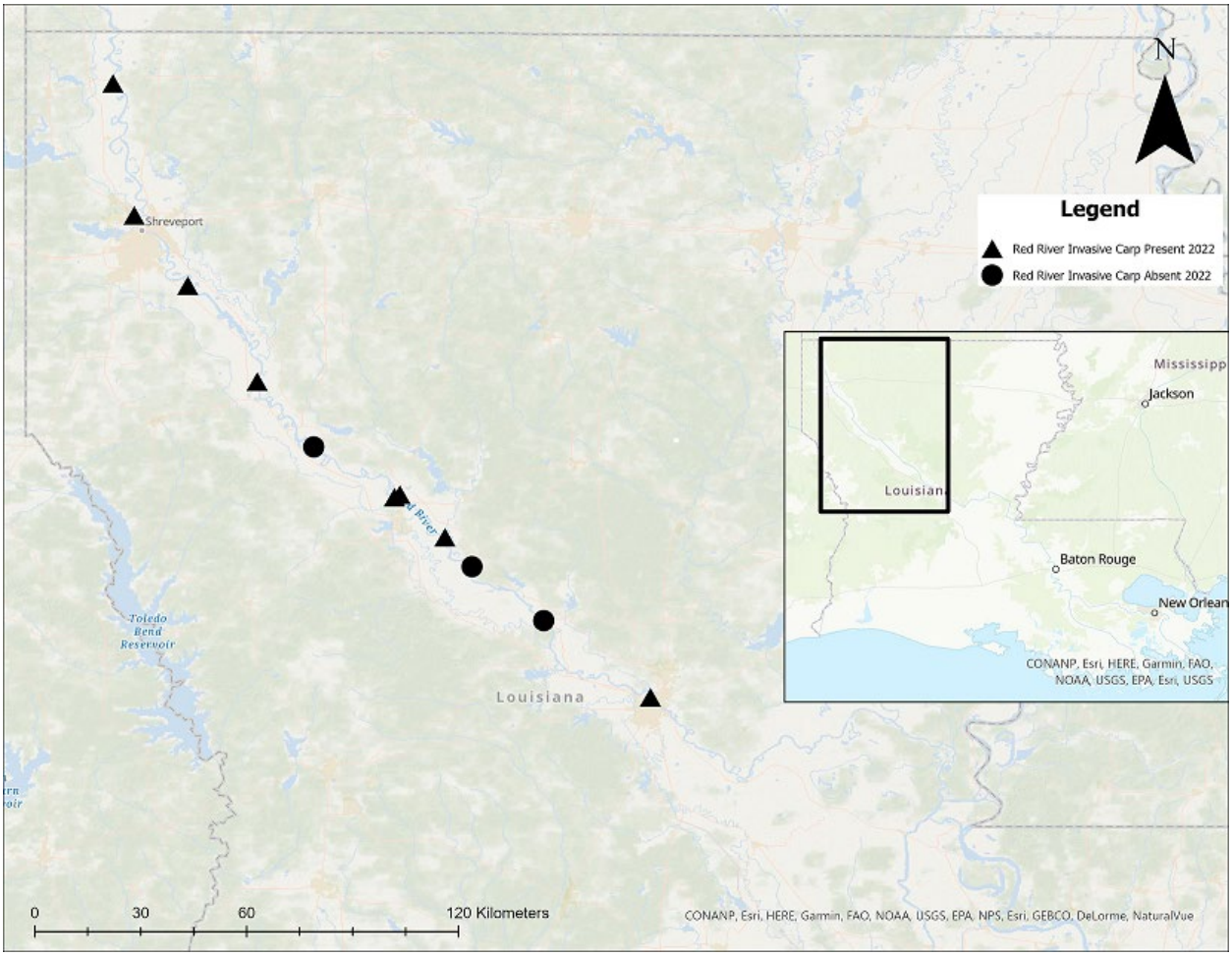

Figure 1. Sample site map indicating sites where invasive carp were present (▲) and those were invasive carp were not detected (●) during 2022 sampling.

Oklahoma:

Ichthyoplankton Tows.—A total of 62 ichthyoplankton tow samples resulted from 12 sampling efforts on the Red River and its tributaries (Figure 2) during the summer of 2022. Dates and location data are listed in Appendix 2.

Light Trapping.—Light trapping conducted during 10 separate sampling events resulted in a combined total of 92 trap-nights on the Red River and its tributaries (Figure 3). Dates and location data are listed in Appendix 3.

qPCR.—In December 2022, aliquots of ethanol from 44 ichthyoplankton tow samples and 99 light trap samples were sent to the Whitney Genetics Lab for qPCR analyses to be completed in spring 2023. These samples were selected out of the 154 total samples as greatest priority by having the greatest quantities of fish and/or debris. The remaining 20 samples will be processed through sorting and identification only.

Sorting and identification.—As of January 2023, the OKFWCO has completed the sorting process for 21 ichthyoplankton samples. Sorting will continue until qPCR results are available. Most light trap samples will not require any sorting prior to the identification process.

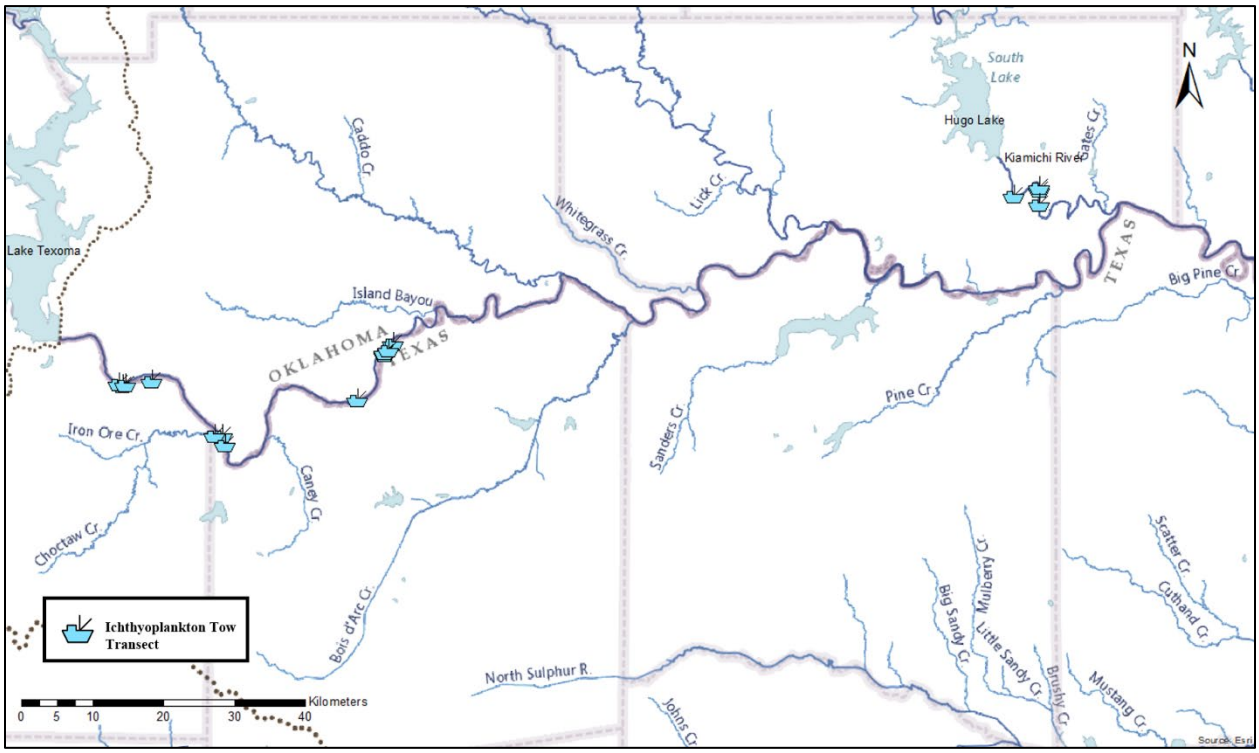

Figure 2. Locations of ichthyoplankton tow sampling completed by the OKFWCO on the Red River and its tributaries below the Denison Dam in Oklahoma and Texas. Ichthyoplankton tow transects are denoted by blue boats.

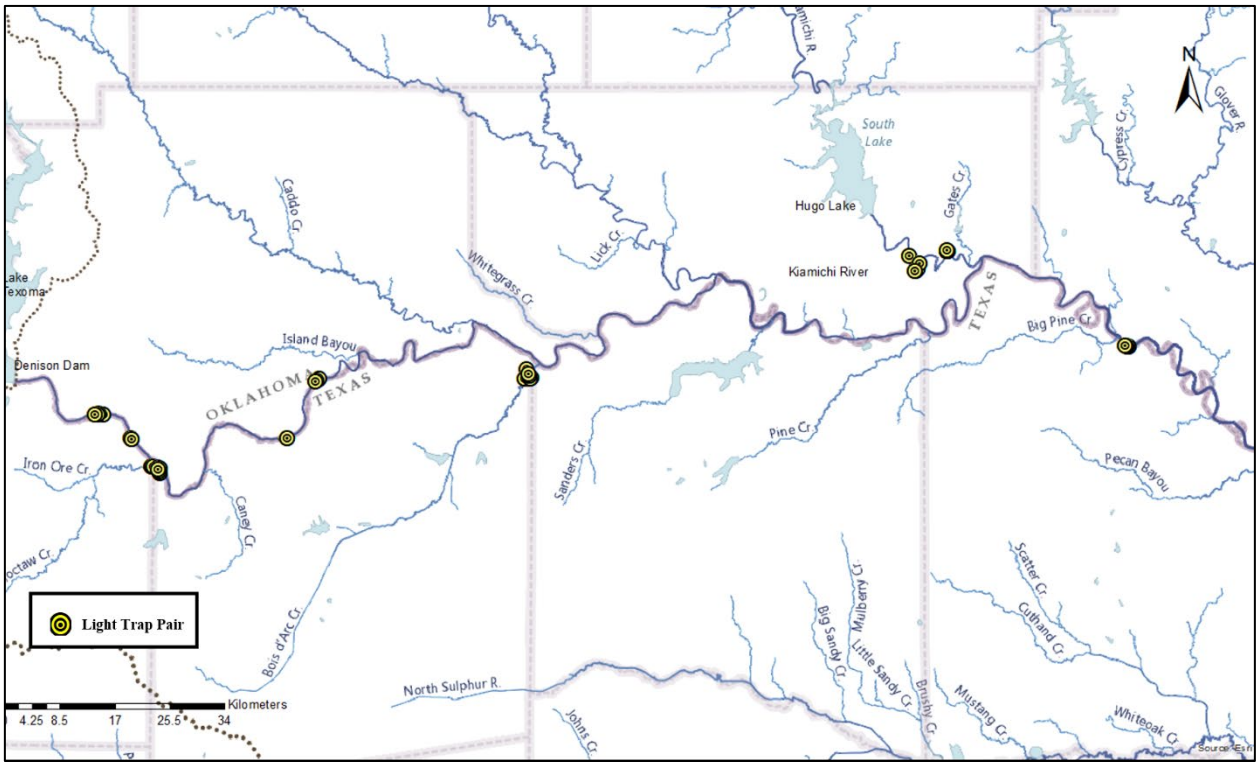

Figure 3. Locations of light trap sampling completed by the OKFWCO on the Red River and its tributaries below the Denison Dam in Oklahoma and Texas. Yellow targets indicate locations of light traps set in tandem.

Recommendation:
Future ichtyhoplankton studies could be carried out at fewer sites with more frequent sampling to ascertain what environmental factors may be triggering reproduction. Sampling during those months not sampled in 2022 may inform when peak reproduction occurs. Similar studies in locations other than the Red River may inform future Red River studies and management.

The increase in numbers of invasive carp larvae sampled from 2021 to 2022 indicate that invasive carp may be expanding and increasing reproductive output in LA. Invasive carp were found at all of the most northern sample sites in LA which increases the liklihood that they are currently, or will soon be, reproducing in the upstream states.

The locations where invasive carp are present suggest that the installation of a barrier at a lock structure would not be an effective management action on the Red River in LA without additional removal or control efforts.

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APPENDIX 1. LDWF ichthyoplankton sample site locations that were sampled during the summer of 2022. Each month consisted of three tows per site.

River	Station	Latitude	Longitude
Red	Hwy 2	32.891829	-93.82062
Red	I-220	32.556355	-93.76662
Red	South	32.377796	-93.63042
Red	Pool 4 - Coushatta 1	32.13402	-93.45382
Red	Pool 4 - Red Oak 2	31.96419	-93.31013
Red	Pool 3 - Grand Ecore 1	31.840176	-93.10468
Red	Pool 3 - Grand Ecore 2	31.84683	-93.09094
Red	Pool 3 - Hamptons 3	31.73833	-92.97624
Red	Pool 3 – Mongomery 4	31.660381	-92.90722
Red	Pool 3 – Colfax 4	31.523226	-92.72458
Red	-	31.331832	-92.45357

APPENDIX 2. Ichthyoplankton tow transects (N = 12) completed by the OKFWCO during summer 2022 on the Red River and its tributaries below Denison Dam in Oklahoma and Texas.

River/Tributary	Date	Latitude	Longitude
Red River	5/3/2022	33.77359	-96.49277
Red River	5/12/2022	33.77297	-96.49282
Choctaw Creek	5/18/2022	33.71926	-96.37068
Kiamichi	5/26/2022	33.97292	-95.36507
Kiamichi	5/26/2022	33.97844	-95.33286
Kiamichi	6/13/2022	33.96400	-95.33086
Red River	6/15/2022	33.78180	-96.45674
Red River	6/21/2022	33.75550	-96.19415

Red River	6/28/2022	33.77359	-96.48948
Red River	7/5/2022	33.71061	-96.36275
Red River	8/16/2022	33.82042	-96.15404
Red River	8/18/2022	33.82042	-96.15404

APPENDIX 3. Quadrafoil light traps deployed by the OKFWCO during summer 2022 on the Red River and its tributaries in Texas and Oklahoma below the Denison Dam. Each row represents a pair of traps in a tandem set.

River/Tributary	Date	Latitude	Longitude
Choctaw Creek	5/18/2022	33.72136	-96.38186
Choctaw Creek	5/18/2022	33.72128	-96.37982
Choctaw Creek	5/18/2022	33.72039	-96.37328
Choctaw Creek	5/18/2022	33.71952	-96.37088
Red River	5/25/2022	33.75419	-96.19414
Red River	5/25/2022	33.75435	-96.19367
Kiamichi River	6/1/2022	33.96907	-95.27788
Kiamichi River	6/1/2022	33.969519	-95.27859
Kiamichi River	6/1/2022	33.96943	-95.27834
Kiamichi River	6/13/2022	33.96907	-95.27788
Kiamichi River	6/13/2022	33.96955	-95.27862
Kiamichi River	6/13/2022	33.94607	-95.32233
Kiamichi River	6/13/2022	33.96322	-95.33073
Red River	6/15/2022	33.78134	-96.44856
Red River	6/15/2022	33.78272	-96.45609
Red River	6/15/2022	33.78134	-96.45694
Red River	6/15/2022	33.78128	-96.46088
Choctaw Creek	6/22/2022	33.71423	-96.37085
Choctaw Creek	6/22/2022	33.71611	-96.37231
Choctaw Creek	6/22/2022	33.71803	-96.37264
Red River	6/29/2022	33.75425	-96.41072

Red River	6/29/2022	33.75341	-96.41003
Red River	6/29/2022	33.75257	-96.40916
Kiamichi River	7/6/2022	33.94912	-96.31875
Kiamichi River	7/6/2022	33.94810	-95.31943
Kiamichi River	7/6/2022	33.95485	-95.31710
Kiamichi River	7/6/2022	33.94655	-95.32346
Bois d'Arc Creek	7/20/2022	33.82272	-95.86392
Bois d'Arc Creek	7/20/2022	33.82228	-95.86382
Bois d'Arc Creek	7/20/2022	33.82257	-95.86283
Bois d'Arc Creek	7/20/2022	33.83381	-95.86188
Bois d'Arc Creek	7/20/2022	33.82247	-95.86437
Bois d'Arc Creek	7/26/2022	33.82355	-95.85524
Bois d'Arc Creek	7/26/2022	33.82309	-95.85537
Bois d'Arc Creek	7/26/2022	33.82246	-95.85692
Bois d'Arc Creek	7/26/2022	33.82625	-95.85850
Bois d'Arc Creek	7/26/2022	33.82771	-95.85984
Big Pine Creek	8/3/2022	33.85975	-95.02707
Big Pine Creek	8/3/2022	33.85983	-95.02859
Big Pine Creek	8/3/2022	33.86028	-95.03006
Big Pine Creek	8/3/2022	33.86035	-95.03047
Big Pine Creek	8/3/2022	33.86047	-95.03116
Red River	8/16/2022	33.82170	-96.15065
Red River	8/16/2022	33.82203	-96.15004
Red River	8/16/2022	33.82034	-96.15411
Red River	8/16/2022	33.81915	-96.15455