Final Performance Report

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Assessing pathways of introduction of non-native fishes (Sheepshead minnow: *Cyprinodon variegatus* and Gulf killifish: *Fundulus grandis*) in Texas streams.

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TEXAS PARKS & WILDLIFE

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Location(s):

Brazos River (Middle and Upper Brazos River basin), Texas

Upper Red River Basin (Wichita, Pease and Red River), Texas

Objective(s):

The aim of this study was to evaluate the pathways through which two invasive species Sheepshead minnow (*Cyprinodon variegatus)* and Gulf killifish (*Fundulus grandis)* are introduced into Texas waters by surveying the bait industry in selected basins in Texas and through direct surveys of current populations in the Brazos River and Upper Red River basins where introductions have occurred. This main objective was accomplished by completing four tasks: 1) Description of live bait industry via direct expert opinions and published information; 2) Informal interviews and visits to selected local live bait shops; 3) Surveys of focal species (*C. variegatus* and *F. grandis*) in sites located in the Middle and Upper Brazos River and Upper Red River to quantify abundances, feeding ecology, and functional traits; and 4) Development of a landscape model using an ecological niche modelling (ENM) to map current and potential future locations of introductions of the focal species in other basins in Texas.

Significant Deviation(s): While not representing significant impacts to study progress COVID-19 constraints affected the timing of fish sampling and delivery of stable isotope data as noted below.

Summary of progress

The activities accomplished during this reporting period are listed within each task. All activities involving field and laboratory data collection were completed between Summer 2020 and Summer 2021. Activities for tasks 1-2 and 4 are completed. Task 3 is completed with the exception of the stable isotope analysis. Due to COVID-19 situation there were delays in submitting samples to the SIA laboratory in Georgia and we are still waiting 248 tissue samples and 43 for samples that were submitted for analysis in June 2021.

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EXECUTIVE SUMMARY

Between Summer 2020 and Summer 2021, we conducted seasonal surveys of the live bait industry in Texas using informal phone conversations with bait shop owner, manager and/or employees, internet sources, citizen science website (e.g., iNaturalist), literature review, and conversations with experts to better understand pathways of introduction of the two Cyprinodontiformes, Sheepshead minnow (*Cyprinodon variegatus*) and Gulf killifish (*Fundulus grandis*). Live aquatic bait was, for this survey, defined as fish and/or any other aquatic living organism (e.g., shrimps, worms, etc.). Preserved bait (both aquatic and non-aquatic), while not the focus of this effort, were identified and recorded. In total, 170 bait shops were contacted via informal phone calls every season from a combined list provided by TPWD, using the *baitFinder app*, and internet sources. Out of this number, we visited 61 live bait shops to confirm the species of the live baits being sold at the bait shops. There were 24 fish species identified from bait shop surveys. Based on informal phone surveys and visits to bait shops, we did not find evidence for the two focal invasive species to be sold as baits. Golden shiner (*Notemigomus crysoleucas*) and Fathead minnow (*Pimephales promelas*) were the most common species being sold across all seasons and bait shops and they were sold under the names of extra-large, large, medium, or small minnows. In addition, we contacted 21 live bait businesses including fisheries and pond management businesses, fish hatcheries, and fish farms in or around Texas to learn about the diversity of live bait they produce, distribution of the bait, and their specific knowledge of the Gulf killifish and Sheepshead minnow within the bait industry.

Seasonal field surveys of the populations of focal species were conducted in 16 sites along the Middle and Upper Brazos River and 6 sites in the Upper Red River to document the status of these species in relation to native congeners, Red River pupfish (*C. rubroflubiatilis*) and Plains killifish (*F. zebrinus*). Overall, we documented the presence of Sheepshead minnow in two sites of the Middle Brazos River between Lake Whitney and Possum Kingdom Lake, while the Gulf killifish was collected in two locations in the Brazos River between Possum Kingdom Lake and Lake Waco. Identification of these species was based on morphological and taxonomic characters only. We did not find evidence of the native and invasive cyprinodontids occurring at the same sites. The two native species, the Red River pupfish and Plains killifish, along with other species including the Smalleye shiner (*Notropis buccula*), Sharpnose shiner (*N. oxyrhynchus*), Shoal Chub (*Macrybopsis hyostoma*), Red Shiner (*Cyprinella lutrensis*), Plains minnow (*Hybognathus placitus*), others (Table 11) were commonly found in sites of the Upper Brazos River above Possum Kingdom Reservoir. Except for Smalleye shiner and Sharpnose shiner, all other cyprinids were also found in surveyed sites of the Upper Red River.

Dietary, isotopic, and morphological analyses suggest that 1) high overlap in feeding ecology and morphological space occupied by Sheepshead and Red River pupfish. Red River pupfish also exhibits nearly identical feeding patterns to its potential *Cyprinodon* “hybrids” in the Middle Brazos River; 2) there was less overlap in feeding and morphological space observed between the two species of killifish.

The Ecological Niche Model (ENM) for two invasive fish, Sheepshead minnow and Gulf killifish, using random forest models suggest that the environmental factors most strongly contributing to suitable conditions for invasion included high portions of upstream pasture-land use, lower local elevations, and lower groundwater contributions to base flow. These findings will be validated using data collected from this study.

INTRODUCTION

Introductions of invasive species are widely recognized as significant drivers of ecological change in freshwater ecosystems, with impacts ranging from establishing of new populations (which alter the chemical and physical habitat features, and the trophic structure of aquatic and terrestrial ecosystems; Owsley et al. 2017); to hybridization (Echelle & Connor 1989, Wilde & Echelle 1992) and alteration of ecosystem processes (Atkinson et al. 2010, Scott et al. 2012). Invasive species can become established outside of their native home ranges in a variety of different ways including natural phenomena such as flooding. However, the most common methods of introduction are by human involvement, which might include commercial shipping vessels, recreation (with use of live bait and the subsequent dumping of live bait), biological control (e.g., the use of Western mosquitofish to control mosquitos), aquaculture, the pet and aquarium trade, and accidental escape into natural waters.

Bait-bucket introductions are widespread in freshwater systems in the United States (Keller & Lodge 2007, Kilian et al. 2012), and appear responsible for the introduction of two species of coastal cyprinodontids, Sheepshead minnow and Gulf killifish into inland Texas waters (Hillis et al. 1980, Hubbs et al. 1991, Wilde & Echelle 1992, Hubbs et al. 2008, Cheek & Taylor 2015). Over the past few decades, both species have been reported in the Pecos River drainage in New Mexico and Texas (Hillis et al. 1980, Hubbs et al. 2008, Cheek & Taylor 2015). In the Brazos River drainage, Sheepshead minnow and Gulf killifish have been reported in freshwater habitats ranging from above Possum Kingdom Lake to Lake Whitney (Hubbs et al. 2008, Labay et al. 2013, Wilde 2015) (Fig. 1).

The effects of cyprinodontid introductions have been reported in several studies following the releases. For example, a decline in the abundance of Plains killifish in the Lower Pecos River has been observed after the introduction of Gulf killifish (Cheek & Taylor 2015). The introduction of Sheepshead into the Pecos River in early 1980s (1980-84) coinciding with the range of the endemic Pecos pupfish *C. pecosensis*, resulted in hybridization between these two species (Wilde & Echelle 1992). Within a span of five years, hybrids of Sheepshead and Pecos pupfish were found over half of the native geographic range of Pecos pupfish (Echelle & Connor 1989). The introduction and hybridization of Sheepshead has not been limited to the Pecos River system. In the 1970s, Sheepshead was introduced in Leon Creek, an isolated spring in the Pecos River system, which supports the endemic Leon Spring pupfish (*C. bovinus*; Echelle & Echelle 1992). Hybridization between Sheepshead and Leon Spring pupfish was observed within two years of discovery of Sheepshead in Leon Creek (Hubbs et al. 1978, Echelle & Echelle 1992). Additional introductions of Sheepshead have been documented into Lake Balmorhea (Texas) in 1960s, where it hybridized with the endemic Comanche Spring pupfish (*C. elegans*; Stevenson & Buchanan 1973, Echelle & Echelle 1994). Recently (2011), Sheepshead was found in the Brazos River upstream of Possum Kingdom Lake and may pose a conservation threat for the native Red River pupfish (Wilde 2015).

Sheepshead minnow is native to the coastal Gulf regions of Texas (Echelle & Connor 1989) (Fig. 1a), and its native range is as far north as coastal Massachusetts and as far south as the Yucatan Peninsula, and appears to be extremely resilient to temperature and salinity changes (Hubbs et al. 1991). In Texas, introductions of Sheepshead minnow have occurred the Pecos River system and Upper Brazos River (Fig. 1). Their extreme physiological tolerances may favor Sheepshead minnow to become established in new environments. Red River pupfish similar to other *Cyprinodon* species (Echelle 1973, Pigg et al. 1995). A recent study (Ayers 2018) suggests that although populations of Red River pupfish in the Brazos River expressed intermediate morphological traits inferring hybridization had occurred, molecular analysis did not show signs of hybridization. While conservation efforts for Texas pupfishes have focused on the two federally endangered species, Comanche Spring pupfish and Leon Spring pupfish, as well as the imperiled Pecos pupfish, a better understanding of the introduction sources of live bait and status of the invaders on recipient fish assemblages is crucial for developing effective regulations and educating anglers to reduce bait-related introductions.

The Gulf killifish is native to the coastal waters of the Gulf of Mexico (Fig. 1b), where they can tolerate both low (<10‰) and high (>65‰) salinities (Vaughn et al. 2016, Crego & Peterson 1997). Since the late 1970s, invasive populations of Gulf killifish have been observed in the Pecos, Brazos, and Upper Rio Grande basins in Texas (Hillis et al. 1980, Cheek & Taylor 2015; Vaughn et al. 2016) (Fig. 1). In the Lower Pecos River, Gulf killifish appears to be one of the most dominant fishes in the more upstream areas. Increased populations of Gulf killifish in the Pecos River may have played a role in the recent decline of the native Plains killifish (*F. zebrinus*) (Cheek & Taylor 2015). It is believed that this decline is due to interspecific competition for resources (e.g., food), as Gulf killifish shares a common diet with its native congener (Cheek & Taylor 2015). In addition, Vaughn et al. (2016) suggests that this decline of Plains killifish is linked to direct predation by Gulf killifish during periods of drought and isolation to pools, where Gulf killifish is primarily piscivorous.

With recent reports and collections of Sheepshead minnow from the Middle and Upper Brazos River (Mayes & Wilde 2019) and Gulf killifish from Middle Brazos (Cohen et al. 2012) and Wichita River (TPWD 2016), it is essential to evaluate the status of both native and invasive populations of cyprinodontids to better understand potential ecological risks in native populations. In this study, we used Mayes & Wilde (2019) to define the Middle Brazos as the portion of the Brazos River that extends from the southern edge of Possum Kingdom Lake to Lake Brazos in Waco, Texas; whereas the Upper Brazos comprises the Brazos River upstream from Possum Kingdom Lake with its two major tributaries, Double Mountain Fork and Salt Fork Brazos River.

Over the basis of four seasons including Summer, Fall, and Winter of 2020 and Spring-early Summer 2021, we collected information on status of the live bait industry in Texas to learn about live bait species being sold in selected basins for this study and explore the potential for non-native species being sold via bait-bucket. We used a variety of methodologies that includes direct contact with bait dealers and local visits to bait shops within selected study sites. Using direct field surveys, this study updated the occurrence of the two invasive species (Sheepshead minnow and Gulf killifish) and native species (Red River pupfish and Plains killifish) in sites of the Middle and Upper Brazos River and Upper Red River basins. Likewise, this study assessed ecological aspects of populations of these four Cyprinodontiformes in reaches of the Middle and Upper Brazos River and Upper Red River basin, using different approaches based on dietary analysis, stable isotope analysis, and morphological trait analysis to make inferences about resource use between native and invasive cyprinodontids and help us to understand the potential ecological risks of these two invasive species in inland ecosystems. Finally, we constructed an Ecological Niche Model (ENM) for the two invasive Cyprinodontiformes (*C. variegatus* and *F. grandis*) using random forest models with records from Fishes of Texas and 22 predictor variables from the National Hydrography Dataset (NHD 2019) to map out the potential and future locations of introductions of these species across other basins in Texas.

Surveys that were conducted from Summer 2020 to Summer 2021 along sites in the Brazos and Red River were used to validate predications of the model (validation will be included in final report). Findings from this study will be used in conservation planning by Texas Parks and Wildlife Department for their native Fish Conservation Areas Initiative (Birdsong et al. 2019) and Texas Conservation Action Plan (TPWD 2012).

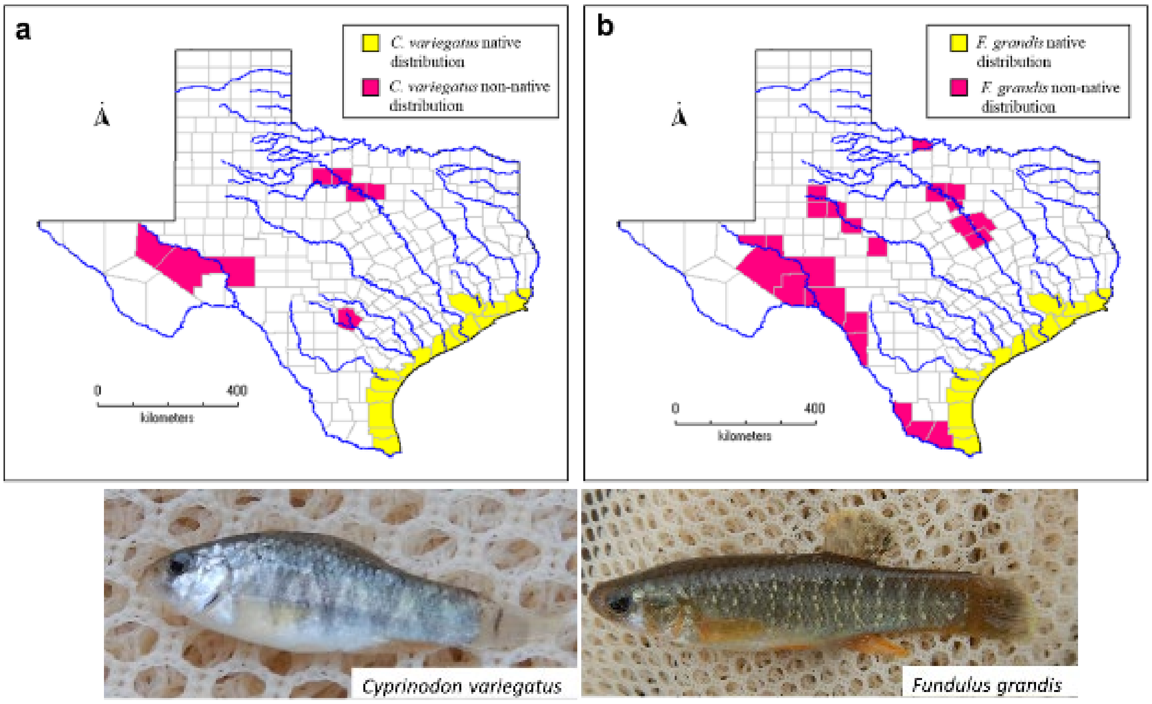

Fig. 1. Distribution in Texas of Sheepshead minnow *C. variegatus* (**a**) and Gulf killifish (*F. grandis*) (**b**) in their native range (yellow) and locations where they have been introduced. Information on species ranges was obtained from USGS and TPWD reports. Photos represent the two targeted cyprinodontid species the *C. variegatus* (left panel) and *F. grandis* (right panel) collected from Brazos River at Mineral Wells HWY 281, 3/12/2021.

Study sites

The Brazos River: The Brazos River basin is the second largest river basin in the state of Texas. Ranging about 1,300 km from the eastern boundary of Stonewall County and emptying in the Gulf of Mexico south of Freeport in Brazoria County, it covers an area of about 110,000 km^2 within the state. The Brazos River is separated into three portions: the Upper, Middle, and Lower Brazos Rivers. The Upper Brazos River is comprised of the Brazos River and two tributaries, the Salt Fork Brazos River, and the Double Mountain Fork Brazos River. Portions of the Brazos River that are located upstream of Possum Kingdom Lake (Palo Pinto County) are in the Upper Brazos River (Mayes et al. 2019). The Middle Brazos River extends from the southern edge of Possum Kingdom Lake to Lake Brazos in Waco, Texas (Mayes et al. 2019). For this study, we surveyed sites along the Middle and Upper Brazos River (Fig. 2). Over the past century, three different impoundments have been built along this stretch of the river:

Possum Kingdom Lake (1941), Lake Whitney (1951), and Lake Granbury (1969). These reservoirs are popular spots for recreational activities such as boating and fishing. Portions of the Brazos located below Lake Brazos are considered the Lower Brazos River

The Red River: The Red River basin is located to the north of the Brazos River. Ranging a total of 1100 km within Texas from Deaf Smith County to Bowie County, it forms the northern boundary between Texas and Oklahoma (TPWD 2019). The Red River Basin covers an area of about 80,000 km^2 within the state and is characterized by red clay and sandstone formations that harbor large amounts of gypsum and salt, causing the river to be brackish (Frye & Leonard 1963). For this study, our surveys were located in sites in the Upper Red River (main channel) and two tributaries, the Wichita River, the Pease River (Fig. 2).

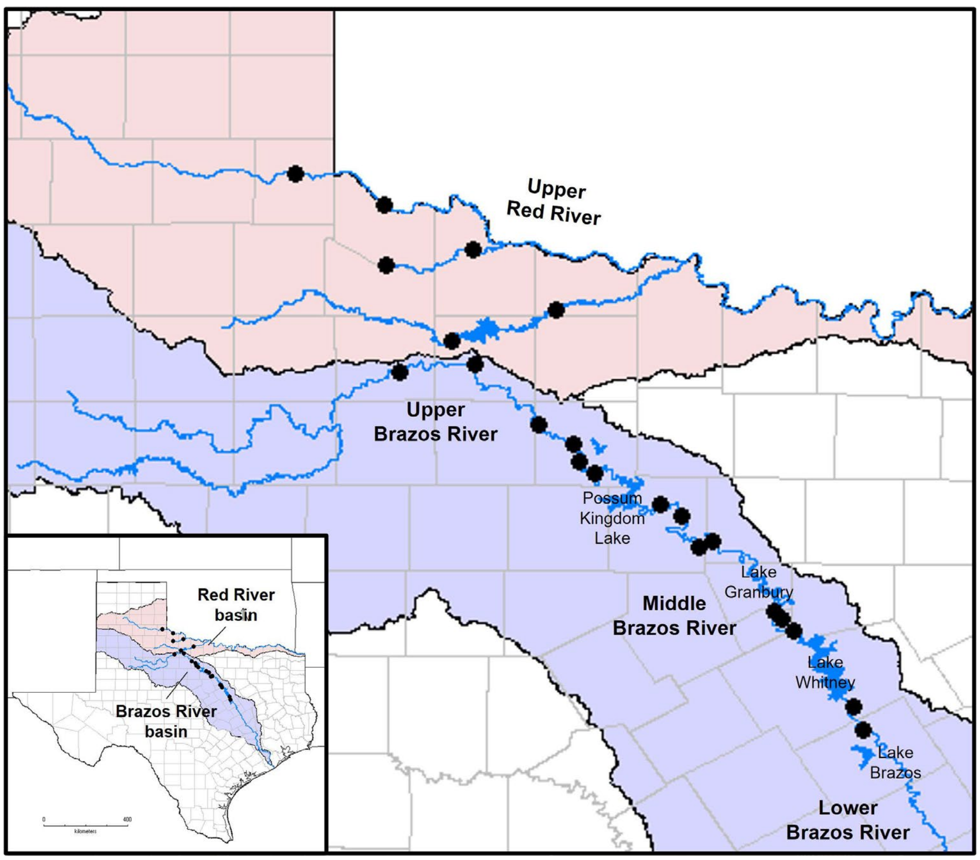

Fig. 2. Map depicting survey locations in the Brazos River and Red River in Texas. The Middle and Upper basin of the Brazos River is depicted in blue and sites (black circles) between upstream Lake Waco and Lake Possum Kingdom are considered Middle Brazos, while sites upstream Possum Kingdom are within the Upper Brazos portion. The Red River basin is depicted in red and sites include those in Red River Channel, Wichita and Pease rivers. Details of sampling sites are provided in Table 9.

In this study, we used fours tasks (described below) to achieve the proposed objectives. Therefore, the description of methods, procedures, results, and discussion will be presented under each proposed task.

TASK # 1. DESCRIPTION OF LIVE BAIT INDUSTRY VIA DIRECT EXPERT OPINIONS AND PUBLISHED INFORMATION.

1.1. Methods and procedures

To assess the status of the live bait industry and learn about the potential of Sheepshead and Gulf killifish being used as inland live baits, we searched for published literature documenting the live bait industry in the state of Texas and compiled expert opinions within the State. Published literature included the Census of Aquaculture, reports by Texas Parks and Wildlife Department (TPWD), the Southern Regional Aquaculture Center (SRAC), and Texas Aquaculture Production at Texas A&M Agrilife Extension and other research papers. We compiled a list of experts from Texas institutions that could provide accurate information of the live bait industry within Texas including Texas aquaculture centers, fisheries and pond management centers, and regional wholesale businesses. These experts were asked about the diversity of bait fish being sold at local facilities, distribution within the state, bait seasonality, bait producers, and specific knowledge of the Sheepshead minnow and Gulf killifish within the bait industry. Both, published information and expert opinions helped to identify the most common live bait fish being produced for baits, demand of species, distributors, and consumers within the State of Texas.

1.2. Results and discussion

Reports published by the SRAC (2021), Texas A&M Agrilife Extension, and 2018 Census of Aquaculture Vol. 3, suggest that the three
(*Notemigonus crysoleucas*, Fig. 3a)
(*Pimephales promelas*, Fig. 3b),
(*Carassius auratus*)

Golden shiners, Fathead minnows, Goldfish, Threadfin and Gizzard shad (*Dorosoma petenense* and *D. cepedianum*), Green sunfish (*Lepomis cyanellus*), and Bluegill (*Lepomis macrochirus*), along with other fish species that are represented by less demand as the main baitfish production in Texas (Table 1). Our conversations with these live bait experts also suggest that most live baits distributed in the Middle and Upper Brazos and Upper Red River sites are transported from Arkansas to Texas and then distributed by local retailers.

Fig. 3a*.* Fathead minnow (*Pimepahles promelas*) purchased at a bait shop in Grafford, TX. 12 June 2020.

Fig. 3b. Golden shiner (*Notemigonus crysoleucas*) purchased at a bait shop in Grafford, TX. 12 June 2020.

Managers of pond farms and fish hatcheries in Texas were also interviewed via phone calls (Table 1). They confirmed that most of the bait fish species are produced and used within the facility as food for fish production with recreational purposes such as Channel catfish, Blue catfish, Hybrid-striped bass, Largemouth bass (both native and Florida) (Table 2). From the 21 interviewed live bait experts, we found no evidence that the target species: Sheepshead minnow (*C. variegatus*) or Gulf killifish (*F. grandis*), are being sold or produced as part of the live bait industry in Texas (Table 2). However, more than half of the live fish bait (8/12 species) reported were non-native to the State of Texas. There was only one interviewee (the hatchery Manager at A.E. Wood Fish Hatchery) that reported the presence of the Sheepshead minnow in surrounding areas of Possum Kingdom Lake.

A report produced by Green (2007) investigated the bait fish types used by sport-boat anglers in eight major bay systems along the Texas coast from May 1995 and May 1996. This document suggests that the Gulf killifish (*F. grandis* but also striped killifish *F. similis*) among other coastal species (e.g., Mullet [*Mugil* sp.] and Atlantic croaker [*Micropogonias undulates*]) were used as live baits on bay-pass private boat trips. Green's study also indicates that the killifish was the third most used live bait in the Sabine Lake during the study period. Similarly, Green (2007) reported an increase of the use of live bait fish along Texas coast on bay-pass sport-boat trips since 1983 to 1996, with most of the increase attributed to the emergence of Atlantic croaker as an effective bait for catching spotted seatrout in the early 1990s. Although the report does not infer any movements of live fish bait into major freshwater rivers, this could be considered as a potential path for sport fishing to move desirable live fish baits via bay-pass boat trips into other freshwater-costal systems.

The Gulf killifish is sold as live bait to anglers throughout the Gulf of Mexico and South Atlantic States (SRAC 2004). It is particularly desired as live bait for Flounder, Spotted seatrout and Red drum in saltwater systems. Waas (1982) reported that in the early 1970s, the natural supplies of Gulf killifish fell short to the demand and resulted in an interest in raising this species commercially in coastal areas of Texas, as they can be tolerant to changes in oxygen concentration and high temperatures (Wass 1982, Strawn et al. 1986, Pershbacher et al. 1995, SRAC 2004). In Texas, early research experiments of *F. grandis* on ponds culture were conducted by Strawn et al. (1986) and Pershbacher et al. (1995) at Texas A&M University, who showed promise for Gulf killifish baitfish production with careful management and planning. Despite the interest for aquaculture, Texas A&M did not pursue the raising of Gulf killifish. In 2008 up to present, new research in Gulf killifish live bait production continued by researchers at the AgCenter and School of Veterinary at Louisiana State University (LSU) to cover the demand of anglers. Recent reports suggest that the LSU Ag Center in collaboration with Louisiana Department of Wildlife and Fisheries (2010) are promoting the production of the Gulf killifish as live bait for saltwater anglers in Louisiana (Green et al. 2010, Anderson et al. 2013).

Table 1. Live bait businesses contacted via telephone to inquire about live bait production, distribution, consumers, etc. Total businesses contacted were twenty-one of which eighteen were located in Texas, one in Oklahoma, and two in Arkansas.

Business Name	Street Address	City	County	State	Zip Code
A.E. Wood Fish Hatchery	507 Staples Road	San Marcos	Hays	TX	78666
John D. Parker East Texas State Fish Hatchery	900 CR 218	Brookeland	Jasper	TX	75931
Possum Kingdom Fish Hatchery	401 Red Bluff Road	Graford	Palo Pinto	TX	76449
Dundee State Fish Hatchery	16824 FM 1180	Electra	Wichita	TX	76360
Tyler Fish Farms	096 County Road	Ben Wheeler	Van Zandt	TX	75754
Sturm Fish Hatchery & Bait Farm	4913 Highway 36 S	Sealy	Austin	TX	77474
Overton Fisheries Inc.	1 H 45S	Buffalo	Leon	TX	75831
Johnson Lake Management SVC.	106 Posey Road	San Marcos	Hays	TX	78666
Vollmar Pond and Lake Management	N/A	Fredricksburg	Gillespie	TX	78624
Herrmann's Fish Farm	4977 County Road	Robstown	Nueces	TX	78380
Tank Hollow Fisheries	200 W Tank Road	Poteet	Atascosa	TX	78062
Lochow Ranch Pond and Lake Management	7571 N. State Hwy6	Bryan	Brazos	TX	77807
The Bait Barn	2704 Hwy 21	East Bryan	Brazos	TX	77803
Lake Pro, Inc.	5150 Franz Rd Ste 800	Katy	Harris	TX	77493
Texas Pro Lake	N/A	Comanche	Comanche	TX	76442
Boblusk outdoors	210 Hwy 372 N. Ste 103	Whitesboro	Grayson	TX	76273
Pond King, Inc.	5924 US-82	Gainesville	Cooke	TX	76240
Magnolia Fisheries	217 N. Coppell Rd	Coppell	Dallas	TX	75019
Jones Wholesale Bait Inc	5799 Prairie Valley Road	Ardmore	Carter	OK	73401
Anderson Farm	4377 Hwy. 70 West	Lonoke	Lonoke	AR	42086
Frisby Fish Farm	15158 Hwy. 89 S.	Lonoke	Lonoke	AR	72086

Table 2. Fish species reported by the 21 live bait businesses (see Table 1) in the states of Texas, Arkansas and Oklahoma. Report from contacted business identified their live fish bait production into those for stocking, used for live bait and forage (feeder), and live bait for recreational fisheries.

Product Type	Common Name	Species	Number (n=21)	Percent	Status in Texas	Purpose
Fishes	Largemouth bass	*Micropterus salmoides*	15	71	Native	Stocking
	Smallmouth bass	*Micropterus dolomieu*	3	14	Non-native	Stocking
	Striped bass	*Morone saxatilis*	8	38	Native	Stocking
	Channel catfish	*Ictalurus punctatus*	15	71	Native	Stocking
	Blue catfish	*Ictalurus furcatus*	4	19	Native (southeast)	Stocking
	Rainbow trout	*Oncorhynchus mykiss*	8	38	Non-native	Stocking
	Koi carp	*Cyprinus rubrofuscus*	2	10	Non-native	Stocking
	Koi fingerlings	*Cyprinus rubrofuscus*	2	10	Non-native	Live Bait-feeder
	Grass carp	*Ctenopharyngodon idella*	11	52	Non-native	Stocking
	Goldfish	*Carassius auratus*	8	38	Non-native	Live Bait-feeder
	Black salty	*Carassius auratus*	3	14	Non-native	Live Bait-feeder
	Walleye	*Sander vitreus*	1	5	Non-native	Live Bait-feeder
	Bluegill	*Lepomis macrochirus*	6	29	Native	Live Bait-feeder
	Coppernose bluegill	*Lepomis macrochirus purpurescens*	11	52	Non-native	Live Bait-feeder
	Redear sunfish	*Lempomis microlophus*	9	43	Native	Live Bait-feeder
	Black crappie	*Pomoxis nigromaculatus*	4	19	Native (east/central)	Live Bait-feeder
	Threadfin shad	*Dorosoma petenense*	6	29	Native (southeast)	Live Bait-feeder
	Gizzard shad	*Dorosoma cepedianum*	2	10	Native	Live Bait-feeder
	Mozambique tilapia	*Oreochromis mossambicus*	9	43	Non-native	Stocking – Live bait
	Fathead minnow	*Pimephales promelas*	18	86	Native	Live Bait-feeder
	Rosy red	*Pimephales promelas*	2	10	Native	Live Bait-feeder
	Golden shiner	*Notemigonus crysoleucas*	13	62	Native	Live Bait-feeder
Hybrid fishes	Koi/carp hyrid	*Cyprinus* hybrid	2	10	Non-native	Stocking
	Sunshine bass	*M. chryops* x *M. saxatilis*	3	14	Non-native	Stocking
	Saugeye	*S. vitreus* x *S. canadensis*	1	5	Non-native	Stocking
	Hybrid bluegill	*L. macrochirus* x *L. cyanella*	1	5	Non-native	Stocking
Other	Nightcrawlers	Unknown	1	5	Unknown	Live bait
	Crickets	Unknown	1	5	Unknown	Live bait
	Crawfish	Unknown	5	24	Unknown	Live bait
	Bullfrog tadpoles	*Lithobates catesbeianus*	1	5	Native	Live bait

TASK # 2. SURVEY OF THE LOCAL LIVE BAIT SHOP INDUSTRY IN SELECTED BASINS (TELEPHONE CALL SURVEYS AND VISITS).

2.1. Methods and procedures

To document native and non-native species being sold as bait in Texas shops, we contacted locally owned bait shops within the study areas (Middle and Upper Brazos and Upper Red River basins) via informal phone calls and by visiting the shops. First, based on the list provided by TPWD collaborators (Monica McGarrity, TPWD Senior Scientist for Aquatic Invasive Species) which contained over 450 bait dealer business including shrimp dealers, bait dealer business building, bait dealer's individual, bait dealer business vehicles; we were able to clean and reduce the list down to 239 businesses (Table 3). From February 2020 to June 2021, calls were made in an attempt to contact each business seasonally. In some cases, repeated call backs were necessary. Some businesses were out of service, others were closed permanently, and others did not answer. Therefore, the list of businesses was reduced to 81 viable businesses that possessed a bait dealer license with TPWD (Table 3). In addition, we contacted 89 live bait businesses that were found using the *baitFinder app* (https://www.baitfinder.com/) and other internet sources (Table 3). In total, we had 170 live bait businesses in Texas that were contacted via telephone surveys during summer 2020 and late spring early summer 2021 (Table 3 and Fig. 4). During the surveys of these businesses, inquiries were made about current types of live bait being sold, most popular type of live bait being sold at their shops by season, and whether they purchased or harvested the live baits. Types of live bait were classified down to species when possible or placed into broader categories depending on the dealer's taxonomic knowledge. All bait reported by businesses, either live and preserved, were recorded and a complete list is provided (Table 4). Information for the business' website was also recorded when available.

Table 3. Summary of live bait businesses contacted either via telephone informal surveys or direct visits to the live bait shops.

1. Businesses that are reported to have a bait dealer license in selected basins within Texas (reported by TPWD)	239
Businesses eliminated due to repeat information (same name, address, contact information)	-115
Businesses eliminated due to closure or disconnected number	-41
2. Total number of viable businesses contacted that possess a bait dealer license with TPWD	81
3. Additional businesses not reported by TPWD found through internet searches or Baitfinder *App*	89
4. Total number of businesses compiled to contact during each season during the 2020-2021 telephone survey	170

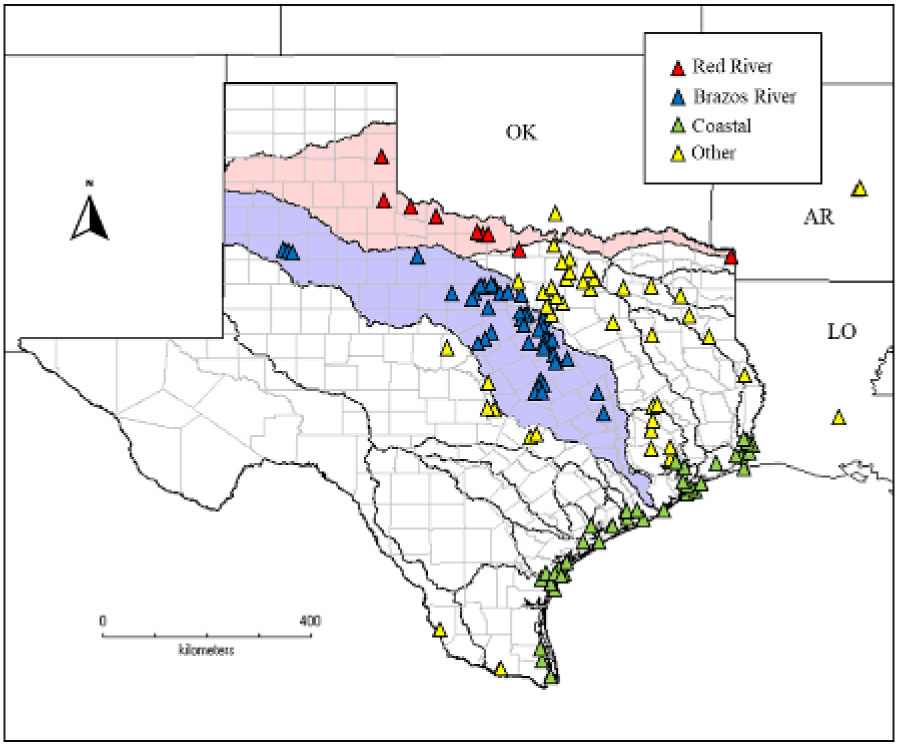

Fig. 4. Map showing the 170 bait shops contacted by phone surveyed during 2020-2021 by region (Red River n=10, Brazos River n=59, Coastal n=58, Other n=44). (GPS coordinates and name of bait shops are provided as Appendix 1).

Locally owned live bait shops located within the study areas were also surveyed via direct visits (Fig. 5). The live bait shops surveyed were randomly selected from a list compiled from several sources including TPWD, internet sources, and the *baitfinder app*. The *baitfinder app* is a smartphone application in which local bait shops publish where they are located and what bait they have available. Select shops were first contacted via the phone and then visited in person to confirm the live fish bait name reported by employees over the phone. In several occasions we purchased the live baits and brought them to the Aquatic Laboratory at SFASU to have a better identification of the species (Fig. 5). All bait items (live and preserved, fish and non-fish) being sold at the shops were recorded. Bait fish were identified down to species in-store whenever possible. All purchased specimens were euthanized using clove oil and preserved in 10% formalin. To account for seasonality of bait types, we visited selected shops during each season throughout the year.

Fig. 5. Live bait shop facilities visited in Brazos River for bait surveys. Photos **a**- **b** corresponds to a live bait shop located in Cleburne, TX and Whitney, TX; **c**) the list of live and frozen baits sold at shop in Whitney, TX; **d**) live fish bait (Golden shiner, *Notemigonus crysoleucas)* purchased at shop in Cleburne, TX near Lake Possum Kingdom; and **e-f**) tanks to keep the live fish baits at fish bait near Lake Possum Kingdom (5/10/2021).

From all the information gathered from the live bait surveys throughout the study basins and from contacting live bait businesses via telephone, we estimated the percentage of bait items reported by the stores (following the procedures in Kilian et al. 2014, Gunderson 2019). Because the questions directed to bait shop owners/employees were in the form of both informal conversations and formal survey questions, this allowed to quantify the types of bait being sold as well as bait popularity. Baits were reported by season to investigate potential seasonal changes within the live bait industry (Kilian et al. 2014, Gunderson 2019). For the bait businesses surveyed in person, percentage (%) of stores selling each bait item at the time of visit was reported (Gunderson 2019). Selected bait shops were questioned about where they obtain their live bait from, who is purchasing bait from their stores (local or outside of the area anglers), whether they have knowledge if live baits are used locally, and whether they knew their wholesale distributor. Percentage of stores that reported collecting their own bait versus those who purchased bait from a wholesale distributor in or out-state was estimated (Cohen 2012). Wholesale bait farms were also contacted, and types of bait fish distributed, and distribution range were reported. This information is important for understanding the distribution of live bait around the state of Texas and more specifically within our study areas, but also provide insights into how live bait is being moved around the state of Texas.

A map showing the location of the bait shops and location of reservoirs within the basins was produced (Fig. 6a). In addition, locations where the invasive species have been collected was added to the map to make inferences about the potential risk of invasions (Fig. 6b, Table 4). This map provides insight into where bait fish are being sold around the study basins and where the invasive species have been collected. If the invasive species is reported to have been found within select bait shops and they are located close to areas where that species has been reported, we could infer that the live bait industry is a potential pathway of introduction of the focal species.

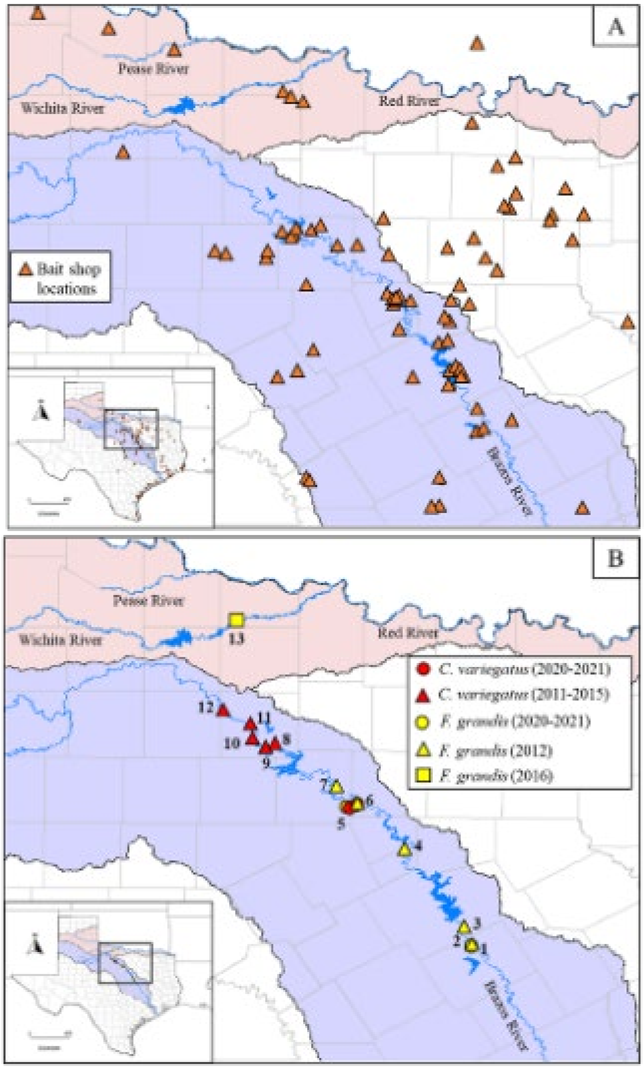

Fig. 6. Locations of the surveyed live bait shops in the Middle and Upper Brazos River and Upper Red River (**a**) in Texas and the locations where the two invasive species *C. variegatus* and *F. grandis* have been reported by Cohen et al. (2012), G. Wilde (2011-2015), report by TPWD (2016), and surveys from this study (2021-2021) (**b**).

Table 4. Locations in the Brazos River and Wichita River (Texas) where the two invasive species, Sheepshead minnow and Gulf killifish, have been reported. The site numbers correspond with those presented in Fig. 5. The year of collections, collectors, and abundance of the species per site are given.

Site	Source	Years	Location	Waterbody	County	Latitude	Longitude	Sheepshead (*C. variegatus*)	*Gulf killifish* (*F. grandis*)
1	Montana et al.	2020-21	at Patrick Rd N. of Rock Creek	Brazos River	McLennan	31.692279	-97.249558		7
2	Cohen et al.	2012	at RV Park	Brazos River	McLennan	31.695000	-97.237700		6
3	Cohen et al.	2012	SH 2114	Brazos River	Hill	31.812700	-97.297100		23
4	Cohen et al.	2012	Between Mitchell Bend and Abby Bend	Brazos River	Hood	32.336800	-97.701900		38
5	Montana et al.	2020-21	at HWY 281 Mineral Wells	Brazos River	Palo Pinto	32.641443	-98.100967	48	10
6	Cohen et al.	2012	at IH20	Brazos River	Parker	32.668700	-98.032600		13
6	Montana et al.	2020-21	at IH20	Brazos River	Parker	32.668700	-98.032600	7	
7	Cohen et al.	2012	at Pleasant Valley Road	Brazos River	Palo Pinto	32.756300	-98.164000		65
8	Wilde	2009-17	at HWY 67 S. of Graham	Brazos River	Young	33.024400	-98.645000	216	
9	Wilde	2009-17	at N. of South Bend	Brazos River	Young	33.024470	-98.645000	38	
10	Wilde	2009-17	at HWY 1287 S. of Graham	Brazos River	Young	33.055630	-98.581100	281	
11	Wilde	2009-17	at HWY 380 W. of Newcastle	Brazos River	Palo Pinto	33.176110	-98.755700	9	
12	Wilde	2009-17	at HWY 79 E. of Elbert	Brazos River	Throckmorton	33.271870	-98.930900	415	
13	TPWD	2016	at SH 25	Wichita River	Wichita	33.869300	-98.839300		9

2.2. Results and discussion

In total, 286 live bait shops were successfully contacted via telephone surveys and 60 were visited (Table 5) within the study area.

Table 5. Number of live bait businesses contacted either via phone or visits by seasons.

Season	Source	Successful Calls	Store Visits
Summer 2020	TPWD list	53	22
Summer 2020	Internet list	71	
Fall 2020	TPWD list	44	18
Fall 2020	Internet list	25	
Winter 2020	TPWD list	2	
Winter 2020	Internet list	4	
Spring 2021	TPWD list	61	20
Spring 2021	Internet list	26	

Telephone survey

In response to questions regarding the type of live bait being sold, no bait shops reported their bait types using scientific names, some had accepted common names, while others had generic names, for example, for Golden shiner and Fathead minnow, the most common names used were depending on body size of the bait (extra-large minnows, large, medium, and small minnows) (Table 6). Some of these generic names were confirmed during visits to the shops within the study area. In total, 24 types of live fish bait were reported by bait shops based on common names (Table 6). One of the 24 types of fish was reported as mudminnow (killifish) from bait shops in the coastal area of Texas and based on the description provided by the bait shop reporter, it suggested that the species was *F. grandis*, which is a prized bait for marine anglers. None of the live bait shops reported Sheepshead minnow (*C. variegatus*) in their bait businesses. In addition, live bait shops reported other types of live baits including five (5) types of crustaceans (e.g., shrimps, grass shrimp, sea-lice, crawfish, and crab), leeches, two types of worms (e.g., earthworms and nightcrawlers), crickets, and salamanders (Table 6).

Table 6. Live bait items reported during the telephone surveys with common names reported by owner/employee, suspected species identification based on in-person surveys and online data bases (TPWD, fishing guides), and status. in Texas of listed live baits.

Bait Type	Common Name Reported	Suspected Species	Confirmed via in-person surveys	Status in Texas within study basins
Fishes	Mudminnow	*Fundulus grandis*		Native (coastal)
	XL minnows	*Notemigonus crysoleucas/ Pimephales promelas*	X	Native
	Large minnows	*Notemigonus crysoleucas/ Pimephales promelas*	X	Native
	Medium minnows	*Notemigonus crysoleucas/ Pimephales promelas*	X	Native
	Small minnows	*Notemigonus crysoleucas/ Pimephales promelas*	X	Native
	Golden shiner	*Notemigonus crysoleucas*	X	Native
	Fathead minnow	*Pimephales promelas*	X	Native
	Goldfish	*Carassius auratus*	X	Non-native
	Black salty	*Carassius auratus*	X	Non-native
	Croaker	*Micropogonias undulatus*		Native (coastal)
	Mullet	*Mugil* spp.		Dependent
	Shad	*Dorosoma* spp.		Native
	Rosy red	*Pimephales promelas*	X	Native
	Perch	*Lepomis* spp.	X	Dependent
	Bluegill	*Lepomis macrochirus*	X	Native
	Green sunfish	*Lepomis cyanella*	X	Native
	Redear sunfish	*Lepomis microlophus*		Native
	Piggy perch	*Orthopristis chrysoptera*		Native (coastal)
	Red shiner minnow	*Cyprinella lutrensis*		Native
	Redhorse minnow	*Cyprinella lutrensis*		Native
	Bream bait			Unknown
	Crappie minnows	*Notemigonus crysoleucas/ Pimephales promelas*	X	Native
	Spottail shiner			
	Chubs			
Crustaceans	Shrimp			
	Grass shrimp			
	Sealice	*Squilla empusa*		Native
	Crawfish			
	Crab	*Callinectes sapidus*		Native
Other	Worms/nightcrawlers	*Lumbricus* spp.		Dependent
	Leeches			
	Crickets			
	Salamanders			

The two most common live baits reported by telephone interviews were Golden shiner (*N. crysoleucas*) and Fathead minnow (*P. promelas*). This result is not surprising giving that these are the two most important baitfish produced nationally (USDA Aquaculture Census 2013, 2018). The Golden shiner and Fathead minnow are mainly raised on farms in Arkansas and shipped throughout the United States. In the 2018 USDA Aquaculture census, production of Golden shiner from 22 farms in Arkansas was worth $13,537 million in sales, while 21 farms of Fathead minnow generated $7,771 million in sales (USDA 2018). These two species are sold not only for live bait use but also as a feeder fish (forage) as suggested by live bait businesses (Table 2) in Texas, Oklahoma and Arkansas. Goldfish (*C. auratus*) and perch (identified as Bluegill sunfish, *Lepomis macrochirus*) were the third and fourth more sold live baits among all species reported (Table 7). The diversity of live bait being sold at the shops also varied depending on the season and region of Texas the shops was located in (Fig. 7, Table 7). For instance, mudminnows was reported from 22 of the 58 bait shops telephone surveyed in coastal businesses (Fig. 7). The Golden shiner, Fathead minnow, Goldfish and perch were consistently sold across seasons and locations (Fig. 7, Table 6). However, bait shops within the Brazos River region were the ones that reported Golden shiner and Fathead minnow as the most common live bait being sold (Fig. 7). Worms were sold throughout the year and all locations. Other live fish baits such as croaker (*M. undulatus*), mullet (*Mugil* sp.), and piggy perch (*Orthopristis chrysoptera*) were reported from coastal bait shops only, where they are the most used baitfish by marine anglers (Green 2007). Crustaceans were an important live bait in coastal shops across all seasons, with shrimps and crabs being the most sold. Shrimps were also reported in some bait shops within the Brazos River basin. However, the percentage of shops reporting shrimps was lower compared to coastal areas (Fig. 7).

Table 7. List of all the live bait items reported during the telephone surveys among seasons. Both, total number of bait shops contacted by season is presented. Live baits were compiled into three categories: fish, crustacean, and other. The number of times reported (and percentage) by season was recorded from each bait shop.

Bait Type	Bait Reported	Total number of stores carrying item (n=170)	Season							
			Summer (n=143)		Fall (n=69)		Winter (n=6)		Spring (n=87)	
			Number	Percent	Number	Percent	Number	Percent	Number	Percent
Fishes	Mudminnow	22	18	13	4	6	0	0	14	16
	XL minnows	4	6	4	4	6	0	0	1	1
	Large minnows	57	42	29	20	29	0	0	22	25
	Medium minnows	27	12	8	7	10	0	0	21	24
	Small minnows	71	61	43	16	23	4	67	25	29
	Golden shiner	34	31	22	9	13	2	33	3	3
	Fathead minnow	27	27	19	5	7	2	33	1	10
	Goldfish	40	45	31	13	19	2	33	9	10
	Black salty	11	10	7	2	3	2	33	5	6
	Croaker	32	23	16	15	22	0	0	18	21
	Mullet	27	17	12	13	19	0	0	16	18
	Shad	10	8	6	0	0	0	0	2	2
	Rosy red	6	9	6	1	1	0	0	2	2
	Perch	35	28	20	6	9	0	0	10	11
	Bluegill	7	7	5	0	0	0	0	0	0
	Green sunfish	2	2	1	0	0	0	0	0	0
	Redear sunfish	2	2	1	0	0	0	0	0	0
	Piggy perch	4	0	0	0	0	0	0	4	5
	Red shiner minnow	1	0	0	0	0	0	0	1	1
	Redhorse minnow	1	2	1	0	0	0	0	0	0
	Bream bait	1	2	1	1	1	0	0	1	1
	Crappie minnows	2	2	1	0	0	0	0	0	0
	Spottail shiner	1	2	1	0	0	0	0	0	0
	Uknown chubs	1	1	1	0	0	0	0	0	0
Crustaceans	Shrimp	52	46	32	20	29	2	33	23	26
	Grass shrimp	2	2	1	0	0	0	0	0	0
	Sealice	3	0	0	0	0	0	0	3	3
	Crawfish	7	3	2	0	0	0	0	4	5
	Crab	17	5	3	3	4	0	0	0	17
Other	Worms/ nightcrawlers	83	58	41	21	30	4	67	30	34
	Leeches	1	1	1	0	0	0	0	0	0
	Crickets	1	0	0	0	0	0	0	1	1
	Salamanders	1	1	1	0	0	0	0	0	0

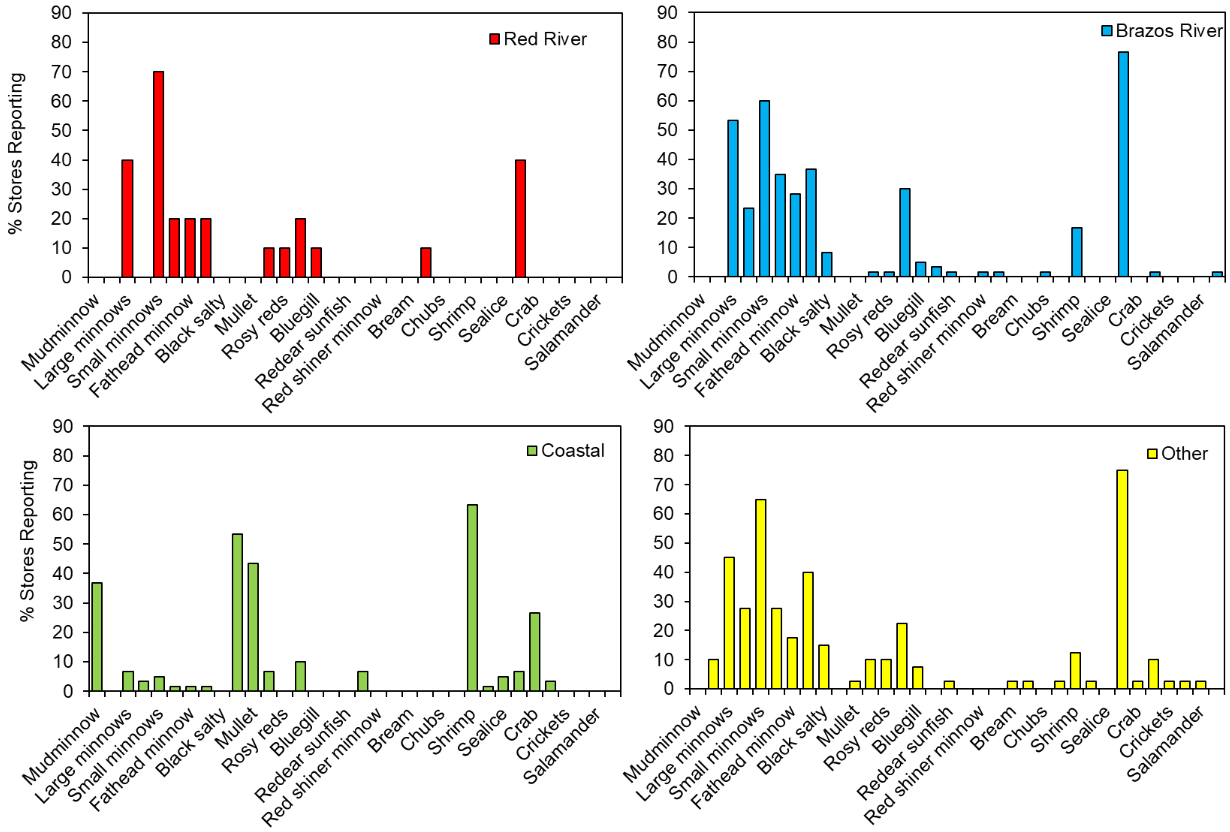

Fig. 7. Live bait types reported by telephone surveys according to four regions: Red River, Brazos River, Coastal and Other [refers to those bait shops located somewhere out of the three regions provided, see Fig. 4]. The number of baits reported by the bait shops are presented as percentage (%).

Bait shop visit surveys

We conducted 61 site visits to live bait shops within selected study area (Table 8). On these visits we observed what baits were present and asked about baits that were sold but not available on that day and baits that were sold all year-round or by seasons. We also asked about specific live baits such as Sheepshead minnow and Gulf killifish. Because in most cases the bait owner/employee did not have knowledge about these names (Sheepshead minnow and Gulf killifish), we had printed pictures of these two focal species to show store employees. Most of the bait shop visited in Brazos River (n= 59) were located between Waco and Graham (McLennan, Hill, Hood, Johnson, and Palo Pinto counties, TX) (Table 8). This is a region of the Brazos River basin that contains a large number of reservoirs that are used for fishing and recreation purposes. In the Upper Red River, only two were visited within adjacent study sites.

Every bait shop we visited within our study area sold Golden shiner and Fathead minnows (both species were called minnows: XL-large, large, medium and small minnows) and this pattern was consistent through all seasons that we visited the bait shops (Table 8, Fig. 8). Goldfish, perch (Bluegill or Green sunfish), shrimps, worms (red worm and nightcrawlers) were also sold as live bait. We also made notes on the type of preserved baits that the stores were selling. When screening the telephone survey calls for bait shops within the study area and the site visits, there was a consistent pattern in the type of live baits being reported. Also, the site visit data seem reliable to us, based as they are on conversations where the bait sellers often provided corroborating details of what we knew from online sources, telephone calls, and wholesale distributers.

Visited bait shops also reported that most of their live baits are purchased from bait distributers and sold to local fishermen, anglers, and tourists visiting the local lakes and rivers. Some of the bait shops informed that the live baits, primarily Golden shiner and Fathead minnows, are brought from Arkansas and distributed to them by local retailers. During our visits to the study area, we had conversations with some fishermen visiting Lake Whitney (11/21/2020, 3 anglers questioned), Possum Kingdom Reservoir (5/10/202, two anglers questioned) and Brazos River at HWY 281 (5/25/2020, 4 local fishermen questioned) to inquire about what they were fishing for and the baits used. All questioned fishermen were using Golden shiner as live bait. They also said that live baits were purchased from local bait shops. When fishermen were asked whether they knew or used Sheepshead minnow or Gulf killifish as live bait, they responded that they were not familiar what these fish and they usually get their live baits from local shops.

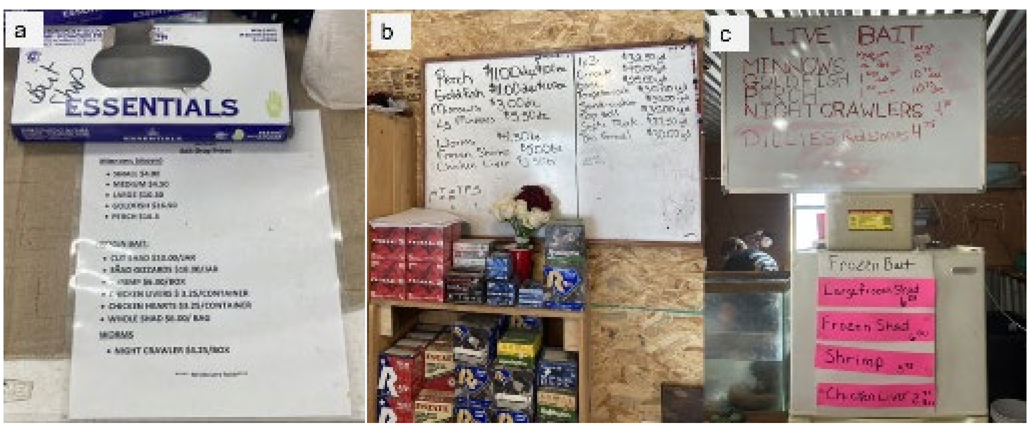

Fig. 8. Live bait species sold in bait shops within Brazos River. **a**) PK-One stop bait shop (Graford, Palo Pinto County, 5/10/2021), **b**) Lucky Minnow Outdoor Store, Whitney, Hill County, 11/21/2020, and **c**) Cornerstore Bait and Tackle Shop, Whitney, Hill County, 11/21/2020.

Table 8. List of live bait shops visited during 2020-2021 in study area within the Brazos and Red River basins. Most minnows of different sizes (large, medium, small) were identified *in situ* Golden shiner (*Notemigonus crysoleucas*) and perch was identified as Green sunfish (*Lepomis cyanellus*). Some baits were purchased and take to the laboratory to verify the species name.

Date Sampled	Season	Store Name	County	Latitude	Longitude	Minnows (Large)	Minnows (medium)	Minnows (Small)	Perch (Green sunfish)	Goldfish	Worms	Shrimp	Purchased (Y/N)
						Golden shiner (*Notemigonus crysoleucas*)			Green sunfish (*Lepomis cyanellus*)				
5/21/2020	Summer	B and C North Forty	Palo Pinto	32.92594	-98.42825	X		X			X		
5/21/2020	Summer	Possum Kingdom Tackle company	Palo Pinto	32.89003	-98.46911	X		X			X		
5/26/2020	Summer	CornerStone Bait and Tackle shop	Hill	31.93102	-97.31982	X		X			X	X	
5/26/2020	Summer	Trailway Trading Post	Palo Pinto	32.81471	-98.03867						X		
5/26/2020	Summer	Lake Whitney Marina	Hill	31.98452	-97.36955						X		Y
6/2/2020	Summer	Park Road 33, Caddo, Texas	Palo Pinto	32.72202	-98.66466	X					X		
6/11/2020	Summer	Buba's Country Store	McLennan	31.70133	-97.21651	X							Y
6/11/2020	Summer	Cornerstone Bait and Tackle Shop	Hill	31.93102	-97.31982	X							
6/12/2020	Summer	Wranglers-Gas station	Hood	32.44657	-97.74488	X							
6/12/2020	Summer	Granbury Mobil-Gas Station	Hood	32.44671	-97.74638	X							Y
6/12/2020	Summer	Granbury Live Bait & Minnows	Hood	32.44671	-97.74638	X							
6/13/2020	Summer	Obis' Mart	Hood	32.42880	-97.67533	X							
6/13/2020	Summer	Joe's bait&tackle	Palo Pinto	32.91981	-98.33435	X							
6/13/2020	Summer	Sportmans HQ	Palo Pinto	32.93570	-98.25994	X							
6/13/2020	Summer	B&C liquor_ Live Bait	Palo Pinto	32.92594	-98.42825	X							
6/14/2020	Summer	Morgan's bait	Parker	32.75919	-97.81725	X							
6/14/2020	Summer	Joshua Feed & Pet	Johnson	32.45439	-97.38487	X					X	X	
6/17/2020	Summer	Pease River Supply	Hardeman	34.28858	-99.73767						X		
6/29/2020	Summer	JM Bait and Tackle	Wheeler	35.22296	-100.25328	X		X			X		
7/1/2020	Summer	Bait Shop	Hill	32.44657	-97.74488	X		X					

Table 8. *Continued*.

Date Sampled	Season	Store Name	County	Latitude	Longitude	Minnows (Large)	Minnows (medium)	Minnows (Small)	Perch (Green sunfish)	Goldfish	Worms	Shrimp	Purchased (Y/N)
						Golden shiner (*Notemigonus crysoleucas*)			Green sunfish (*Lepomis cyanellus*)				
7/1/2020	Summer	River Stop	Hill	32.16307	-97.40794	X		X					
7/1/2020	Summer	Chelle's bait	Palo Pinto	32.54944	-98.37033	X		X			X	X	
10/16/2020	Fall	Gas Station	Palo Pinto	32.43023	-97.67948	X			X	X			N
11/21/2020	Fall	Cornerstore Services	Hill	31.93115	-97.31960	X		X	X	X	X		Y
11/21/2020	Fall	Uncle Gus Marina and Resort	Hill	31.87211	-97.40078								N
11/21/2020	Fall	Mr. K Quick Stop	Hill	31.86812	-97.39926	X							Y
11/21/2020	Fall	Bosque Mini Mart	Hill	31.85939	-97.37753	X		X					N
11/21/2020	Fall	Harbor Master Marina	Hill	31.88545	-97.35772								N
11/21/2020	Fall	Lone Star Gas Station	Hill	31.93751	-97.31707						X		N
11/21/2020	Fall	Lamp Post	Hill	31.98329	-97.33814	X							Y
11/21/2020	Fall	Lucky Minnow Outdoor Store	Hill	31.95099	-97.38919	X		X	X	X			Y
11/21/2020	Fall	Lake Whitney Marina	Hill	31.98410	-97.37083								N
11/21/2020	Fall	Gas Station	Hood	32.43987	-97.79400	X				X			Y
11/21/2020	Fall	Live Bait & Minnow at Wranglers	Hood	32.44657	-97.74488	X				X			Y
11/21/2020	Fall	Granbury live bait	Hood	32.44672	-97.74630	X							Y
11/21/2020	Fall	Tommy's gas station 2	Hood	32.43391	-97.77799								N
11/21/2020	Fall	Gas Station	Hood	32.43023	-97.67948								N
11/21/2020	Fall	Tommy's gas station 2	Hood	32.42643	-97.79260								N
11/21/2020	Fall	El Solar	Hood	32.40432	-97.78040	X							Y

Table 8. *Continued.*

Date Sampled	Season	Store Name	County	Latitude	Longitude	Minnows (Large)	Minnows (medium)	Minnows (Small)	Perch (Green sunfish)	Goldfish	Worms	Shrimp	Purchased (Y/N)
						Golden shiner (*Notemigonus crysoleucas*)			Green sunfish (*Lepomis cyanellus*)				
3/12/2021	Spring	Chevron gas station	Hood	32.47383	-97.82790						X		N
4/9/2021	Spring	Tiger Mart	Johnson	32.33499	-97.42191	X		X			X		Y
4/9/2021	Spring	Prime Corner	Johnson	32.31483	-97.40855	X	X	X	X	X			Y
4/9/2021	Spring	Tri Lakes Tackle Town, LLC	Hood	32.44028	-97.75844	X	X	X					Y
4/9/2021	Spring	Live Bait & Minnow at Wranglers	Hood	32.44650	-97.74477	X		X					N
4/9/2021	Spring	Granbury live bait	Hood	32.44671	-97.74638	X							N
4/9/2021	Spring	El Solar	Hood	32.40432	-97.78040	X					X		N
4/9/2021	Spring	Trading Post	Hill	32.13209	-97.47663	X		X					Y
4/9/2021	Spring	River Stop	Hill	32.16307	-97.40794						X		N
4/9/2021	Spring	Lucky Minnow Outdoor Store	Hill	31.95109	-97.38911	X	X		X	X			Y
4/9/2021	Spring	Cornerstore Services	Hill	31.93102	-97.31982	X			X	X	X		Y
4/9/2021	Spring	Bosque Mini Mart	Hill	31.85934	-97.37751	X		X					N
4/9/2021	Spring	Mr. K Quick Stop	Hill	31.86811	-97.39924	X							Y
4/9/2021	Spring	My Stop	McLennan	31.53369	-97.21069	X							Y
4/9/2021	Spring	Rida Corner/ Alexander Express	McLennan	31.56946	-97.16479	X		X					N
5/10/2021	Spring	Trophy bait and Tackle	Palo Pinto	32.80417	-98.15578	X		X	X	X	X		Y
5/10/2021	Spring	The Trading Post	Palo Pinto	32.81481	-98.03886						X		N
5/10/2021	Spring	PK One stop	Palo Pinto	32.89089	-98.45181	X	X		X	X			Y
5/10/2021	Spring	Possum Kingdom Tackle	Palo Pinto	32.89003	-98.46911								N
5/10/2021	Spring	North Forty Live Bait	Palo Pinto	32.92594	-98.42825	X		X	X		X		N
6/25/2021	Summer	My Stop	McLennan	31.53369	-97.21069	X							N
6/25/2021	Summer	Alexander Express	McLennan	31.56946	-97.16479			X					Y

TASK # 3. QUANTITATIVE DESCRIPTION OF SPECIES ABUNDANCE, FEEDING ECOLOGY, AND FUNCTIONAL TRAITS OF *Cyprinodon variegatus* AND *Fundulus grandis*.

3.1. Methods and procedures

For this study, we assessed the population status and ecological aspects of the two invasive species Sheepshead minnow (*C. variegatus*) and the Gulf killifish (*F. grandis*) on sites located in the Middle and Upper Brazos River, Red River and tributaries: Wichita and Pease rivers (Table 9, Fig. 9). In addition to Sheepshead and Gulf Killifish, we assessed the populations of the native Red River pupfish (*C. rubrofluviatilis*) and the Plains killifish (*F. zebrinus*) to examine the potential ecological risk of the two invasive species on the native species.

Table 9. Study site locations sampled within the Brazos and Red River basins, Texas. Site numbers correspond with those presented in Figure 9.

Site	Basin	Location	County	Latitude	Longitude
1	Middle Brazos River	Patrick Road, N. of Rock Creek	McLennan	31.692279	-97.249558
2	Middle Brazos River	Smith Bend, S. of Lake Whitney	Hill	31.812245	-97.297426
3	Middle Brazos River	County Road 1175, S. of Nemo	Somervell	32.204070	-97.606067
4	Middle Brazos River	HWY 67, S. of Lake Granbury	Somervell	32.271600	-97.664349
5	Middle Brazos River	Camp Arrowhead, Cleburne	Somervell	32.305090	-97.703820
6	Middle Brazos River	Riley Ct., N. of Granbury	Hood	32.338542	-97.703175
7	Middle Brazos River	HWY 281, S. of Mineral Wells	Palo Pinto	32.641443	-98.100967
8	Middle Brazos River	Brazos River at IH20	Parker	32.667050	-98.032920
9	Middle Brazos River	HWY 180, Palo Pinto	Palo Pinto	32.797930	-98.186440
10	Middle Brazos River	HWY 4, N. of Worth Ranch	Palo Pinto	32.863442	-98.302365
11	Upper Brazos River	HWY 67, S. of Graham	Young	33.024536	-98.644966
12	Upper Brazos River	HWY 209, S. of Graham	Young	33.081442	-98.728021
13	Upper Brazos River	HWY 380, W. of New Castle	Young	33.176002	-98.755922
14	Upper Brazos River	HWY 79, E. of Elbert	Throckmorton	33.271624	-98.930633
15	Upper Brazos River	HWY 277, Seymour	Baylor	33.579147	-99.267490
16	Upper Brazos River	HWY 267, N. of Rhineland	Knox	33.548179	-99.660506
17	Upper Red River	Wichita River at FM 1919	Knox	33.869300	-98.839400
18	Upper Red River	Wichita River at SH 25	Wichita	33.869300	-98.839400
19	Upper Red River	Pease River at HWY 6 N. of Crowell	Foard	34.095170	-99.728380
20	Upper Red River	Pease River at HWY 283 N. of Vernon	Wilbarger	34.179200	-99.278100
21	Upper Red River	Red River at HWY 6 N. of Quanah	Hardeman	34.414460	-99.735540
22	Upper Red River	Red River at HWY 83 N. of Childress	Childress	34.567020	-100.196010

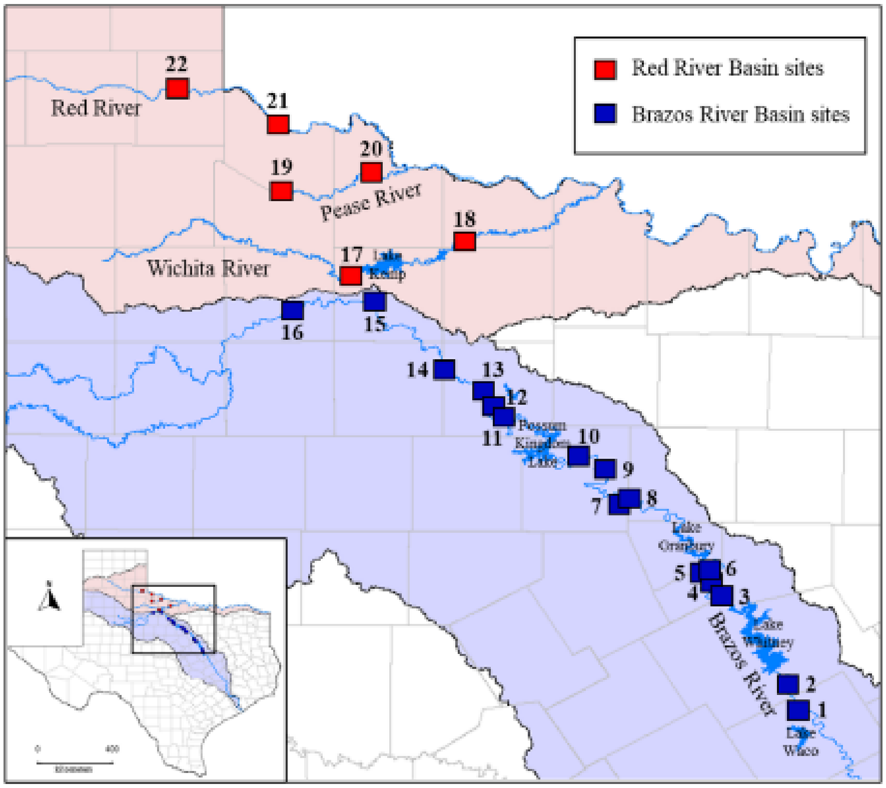

Fig. 9. Locations of study sites within the Brazos River basin (blue) and Red River basin (red), Texas. See Table 9 for specific site locations.

Field surveys

Fish assemblages were sampled at 10 sites along the Middle Brazos River, 6 sites along the Upper Brazos River, and 6 sites in the Upper Red River and its tributaries (Table 9). Sampling sites were selected based on previous collections made by Wilde (2015), Labay et al. (2011), Mayes et al. (2019), and TPWD (2019), where either the invasive focal species or their native congeners had been found previously. Sites were sampled seasonally from Summer 2020-Spring 2021.

At each sampling site, habitat and physicochemical characteristics were measured to assess the quality of the habitat. Habitat characteristics included water velocity (m/s), river depth, canopy cover, substrate composition, bank width, and percentage of algae and woody debris. Physiochemical characteristics included water temperature (˚C), dissolved oxygen (mg/L), conductivity (µs/cm), nitrate concentrations (NO_{3-}) (mg/L), ammonium concentration (NH_{+4}) (mg/L). pH, total dissolved solids (mg/L), salinity(ppt), and turbidity (m/s). Water quality and physical parameters were measured using a portable multiparameter YSI-meter (ProDSS), turbidity meter (Apera TN400 Portable Turbidity Meter), and a current velocity meter (Swoffer Model).

Fish communities were sampled using a seine net (6m long x 1.5m deep). A minimum sampling effort of 10 seine hauls was conducted both upstream and downstream from the access point at each site. However, additional seine hauls were conducted as needed to cover all potential habitat within the sampling reach. Each sampling reach was a minimum ~500 m in length. Once captured, all fish were enumerated. With the exception of the invasive species, all individuals of other species were counted and then released alive back into the river. For the native species, Red River pupfish and Plains killifish, a maximum of 30 individuals were taken from each site, 10 individuals were preserved in ice, 10 individuals were preserved in 90% ethanol, and 10 individuals were fixed in 10% formalin from each species. For invasive focal species all individuals collected were preserved in either ice or 90% ethanol for laboratory analysis of morphology, stable isotopes, and diet.

In the laboratory, individuals were measured for 25 morphological traits (including: standard length, head length, head height, gape width, interorbital distance, eye position, eye diameter, snout length, snout mouth protrusion, body depth and width, peduncle depth, width and length, mouth position, dorsal length and height, anal length and height, caudal depth and length, pectoral length and height, and pelvic length and height) associated with feeding ecology and habitat use (Montaña et al. 2020). Select specimens preserved in either ice or 90% ethanol were used for stable isotope analysis of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) signatures. $\delta^{13}C$ value is assumed to reflect how material from various production sources is assimilated into consumer tissue; $\delta^{15}N$ reflects vertical trophic position of consumer (Montaña et al. 2020). Each specimen was also dissected and its guts were removed to examine stomach contents under a dissection microscope for analysis of diet. Food items were identified to the lowest taxonomic level possible and then grouped into different major food categories (Montaña & Winemiller 2013).

To supplement our relatively small collection of Sheepshead minnows in the Brazos River, we used specimen that were collected previously by Gene Wilde at Texas Tech University (surveys from 2009-2017) and donated to the fish collection at Stephen F. Austin State University. In addition, we borrowed specimens of Gulf killifish (n=44) from the Texas Natural History Collection (TNHC) at the University of Texas, Austin, that were collected by Cohen et al. (2012) in sites along the Brazos River within our study area. These specimens of Gulf killifish were used for analysis of feeding ecology and functional traits.

3.2. Results and Discussion

Fish Abundance

A total of 3163 Red River pupfish, 54 Sheepshead minnow, 909 Plains killifish, and 16 Gulf killifish were collected across all four seasons (Table 10). In addition, we documented a total of 43 different species across the 22 sites from Summer 2020-Spring 2021 (Table 11). The native cyprinodontids, Red River pupfish and Plains killifish, were collected at five sites in the Upper Brazos River basin (Fig. 10) and five sites within Red River basin (Fig. 11). The invasive Sheepshead minnow was collected at two sites in the Middle Brazos River (Site 7 at HWY-281 below Possum Kingdom Lake, Palo Pinto County and Site 8 at IH20 Parker County, Table 10 and Fig 10), while the invasive Gulf killifish was collected at two sites along the Middle Brazos (Site 1 above Lake Waco McLennan County and Site 7 at HWY-281 Palo Pinto County, Brazos River, Table 10 and Fig. 11).

Neither invasive cyprinodontid species were collected at the same site as its native congener during this survey (Fig. 12) suggesting a clear separation on the segments of the Brazos River that appear suitable for occurrence of these populations. For example, the Red River pupfish and Plains killifish primarily occurred in the Upper Brazos and Red River locations, while Sheepshead and Gulf killifish were found inhabiting sites in the Middle Brazos River (Fig. 10 and 11). Historically, the Plains killifish native range stretches into the Middle Brazos River (Poss & Miller 1983) in regions where the Gulf killifish has been recently collected; however, we did not find the two species occurring together. Similarly, the surveys from G. Wilde from 2009-2017 (samples collected by Wilde were used in this study) suggested that Sheepshead minnow occurred at sites in the Upper Brazos River where Red River pupfish was also present. In a thesis by Kristina Ayers (2018), she reported that in a written communication with Gene Wilde (2012), he reported the occurrence of 11 Sheepshead minnow for first time between 2006 and 2010 near Possum Kingdom Lake (middle Brazos River). Likewise, Ayers (2018) reported that in the communication with written Wilde (2012), he reported 32 Sheepshead minnows between August and September of 2011 in the Brazos River South of Graham upstream of the Possum Kingdom Lake with not presence of Red River pupfish. During our surveys (2020-2021), we did not find these two *Cyprinodon* species occurring together in the same locations (Fig. 10).

Table 10. Summary of the sampling locations along with abundance of focal cyprinodontid species collected over the year survey. Numbers listed in brackets indicate individuals preserved for future analysis in the laboratory.

Site	Location	Water body	County (Texas)	*C. rubrofluviatilis*	*C. variegatus*	*F. zebrinus*	*F. grandis*
1	at Patrick Road, N. of Rock Creek	Brazos River	McLennan County	0	0	0	[7]
2	Smith Bend, S. of Lake Whitney	Brazos River	Hill County	0	0	0	0
3	County Road 1175, S. of Nemo	Brazos River	Somervell County	0	0	0	0
4	HWY 67, S. of Lake Granbury	Brazos River	Somervel County	0	0	0	0
5	Camp Arrowhead, Cleburne	Brazos River	Somervell County	0	0	0	0
6	Riley Ct., N. of Granbury	Brazos River	Hood County	0	0	0	0
7	HWY 281, S. of Mineral Wells	Brazos River	Palo Pinto County	0	[47]	0	[9]
8	Brazos River at IH20	Brazos River	Parker County	0	[7]	0	0
9	HWY 180, Palo Pinto	Brazos River	Palo Pinto County	0	0	0	0
10	HWY 4, N. of Worth Ranch	Brazos River	Palo Pinto County	0	0	0	0
11	HWY 67, S. of Graham	Brazos River	Young County	0	0	0	0
12	HWY 209, S. of Graham	Brazos River	Young County	1 [16]	0	[4]	0
13	HWY 380, W. of New Castle	Brazos River	Young County	[1]	0	[3]	0
14	HWY 79, E. of Elbert	Brazos River	Throckmorton County	[1]	0	[10]	0
15	HWY 277, Seymour	Brazos River	Baylor County	564 [61]	0	247 [77]	0
16	HWY 267, N. of Rhineland	Brazos River	Knox County	[42]	0	4 [33]	0
17	Wichita River at FM 1919	Wichita River	Knox County	[14]	0	154 [30]	0
18	Wichita River at SH 25	Wichita River	Wichita County	0	0	0	0
19	at HWY 6 N. of Crowell	Pease River	Foard County	620 [121]	0	165 [79]	0
20	at HWY 283 N. of Vernon	Pease River	Wilbarger County	546 [91]	0	3 [55]	0
21	at HWY 6 N. of Quanah	Red River	Hardeman County	454 [32]	0	2 [25]	0
22	at HWY 83 N. of Childress	Red River	Childress County	573 [26]	0	[4]	0

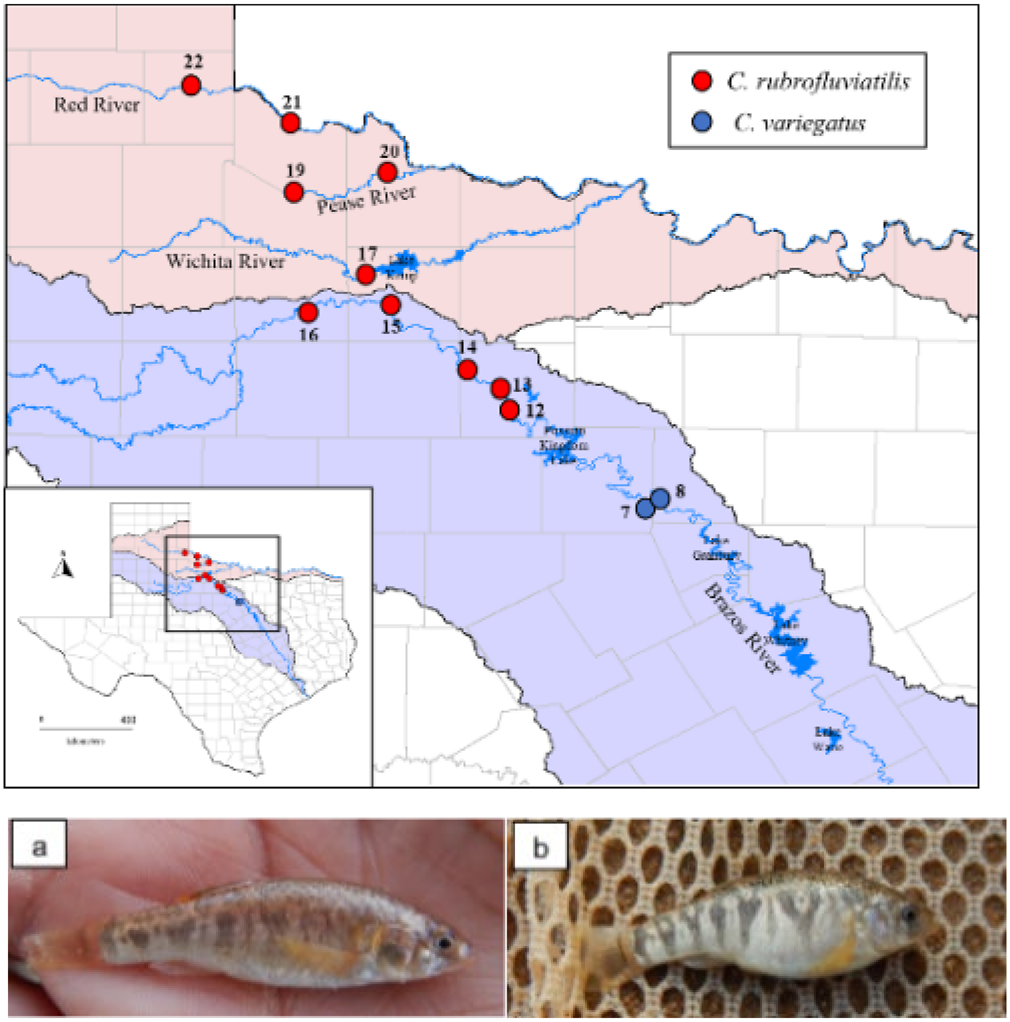

Fig. 10. Locations of the sampling sites along the Brazos River basin (blue shade) and Red River basin (red shade) where the native Red River pupfish (*C. rubrofluviatilis*) and the invasive Sheepshead minnow (*C. variegatus*) were collected during the 2020-2021.
Photos **a** and **b** show the two species of *Cyprinodon* collected within the study area. The native (**a**) Red River pupfish *(C. rubrofluviatilis*) collected in the Brazos River (Site15) and the invasive *(***b**) Sheepshead minnow (*C. variegatus*) collected in the Brazos River at HWY- 281 (Site 7).

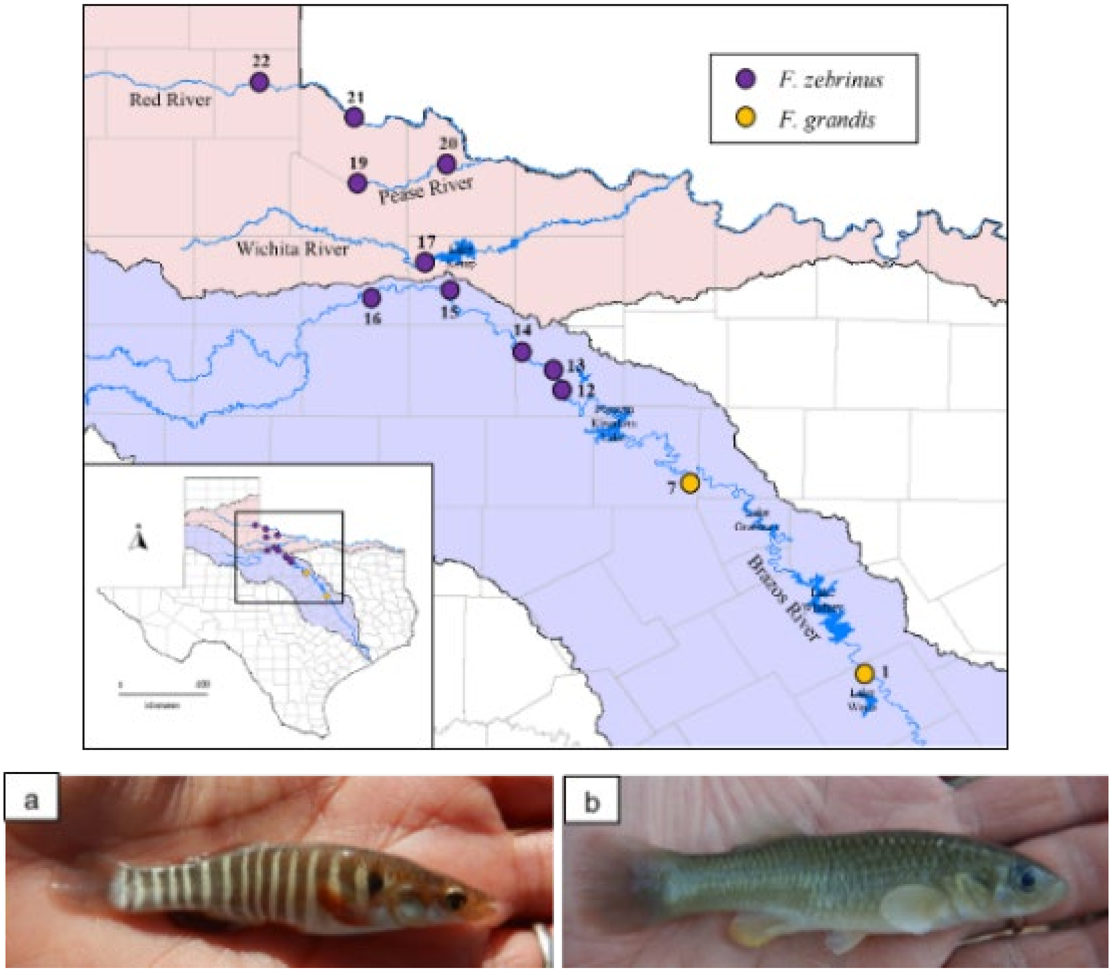

Fig. 11. Locations of the sampling sites along the Brazos River basin (blue shade) and Red River basin (red shade) where the native Plains killifish (*F. zebrinus*) and the invasive Gulf killifish (*F. grandis*) were collected during the 2020-2021.
Photos **a** and **b** show the two species of killifish collected within the study area. The native (**a**) Plains killifish *(F. zebrinus*) collected in the Pease River (Site 19) and the invasive *(***b**) Gulf killifish (*F. grandis*) collected in the Brazos River (Site 7).

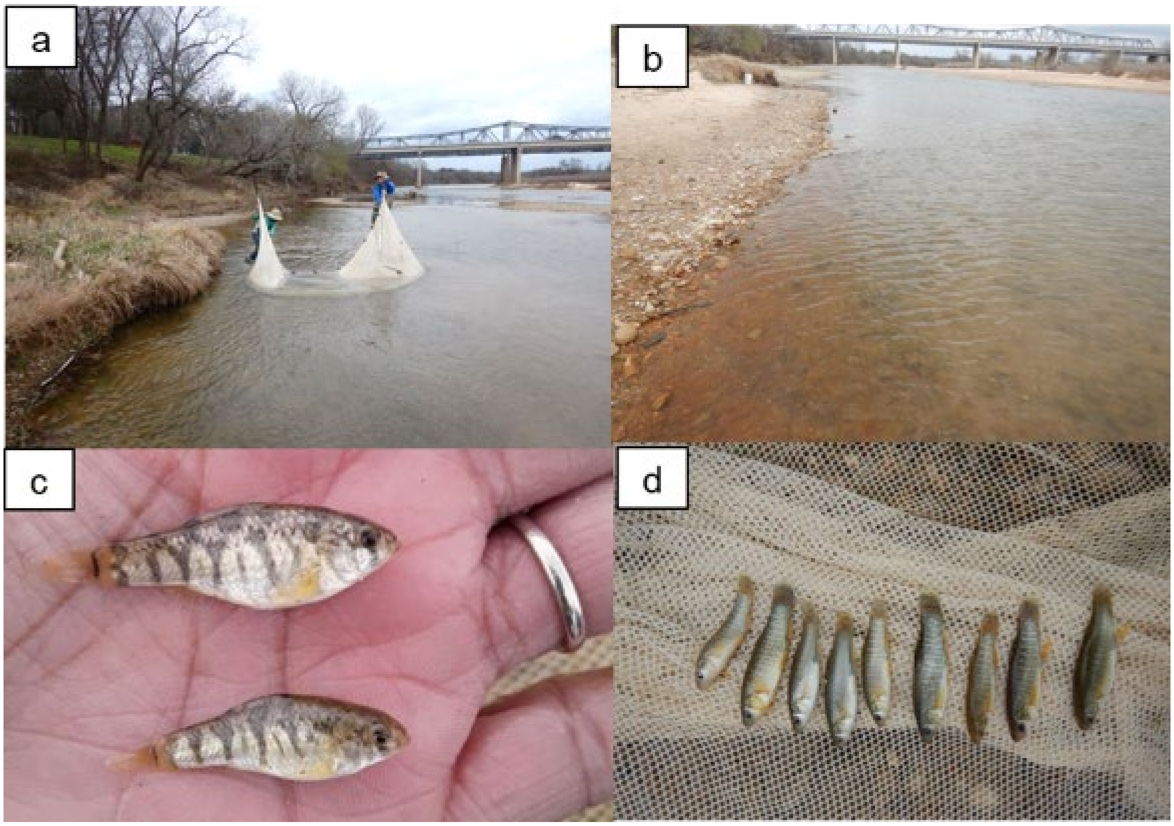

Fig. 12. Location in the Middle Brazos River at HWY 281 (**a**, **b**) South of Mineral Wells (TX) (Site 7) where the two invasive species, Sheepshead minnow(c) and Gulf killifish, were found together. Collections were conducted with a seine (**a**) in the middle channel along shallow habitats, with gravel substrates and lentic waters (**a**, **b**).

Table 11. (Sites 1 – 11). Abundance of fishes collected at each site during all four seasons. Site information found in Table 10. Numbers listed in brackets are specimens preserved and taken to the Fish Collection at Stephen F. Austin State University. All other individuals were counted and released at the collection site.
Note: *Notropis buccula* and *N. oxyrhynchus* were deposited at the Biodiversity Research and teaching Collection at Texas A&M University in coordination with TPWD and USFWS.

			Site										
Family	**Scientific Name**	**Common Name**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Lepisosteidae	*Lepisosteus oculatus*	Spotted gar		1									
	Lepisosteus osseis	Longnose gar				[1]			[1]				[1]
Clupeidae	*Dorosoma cepedianum*	Shad											
Cyprinidae	*Cyprinella lutrensis*	Red shiner			374	639 [88]	10	709 [60]	4701 [56]	2013	53	114 [20]	
	Cyprinella venusta	Blacktail shiner	2209	274 [37]	48	227 [27]	32	209 [25]	719 [1]	463	360	25 [20]	
	Hybognathus placitus	Plains minnow											
	Notropis buccula	Smalleye shiner											
	Notropis oxyrhynchus	Sharpnose shiner											
	Notropis potteri	Chub shiner											[2]
	Notropis bairdi	Red River shiner											
	Notropis buchanani	Ghost shiner											[7]
	Macrhybopsis hyostoma	Shoal chub								51			[4]
	Pimephales vigilax	Bullhead minnow			381	197 [78]	27	538 [9]	936 [5]	514	109	43 [12]	9 [22]
	Machrybopsis australis	Prairie chub											
	Cyprinidae	Juvenile cyprinids											
Catostomidae	*Carpoides carpio*	River carpsucker						[2]	1				[10]
	Ictiobus bubalus	Smallmouth buffalo			1								
Ictaluridae	*Ameiurus natalis*	Yellow bullhead											
	Ictalurus punctatus	Channel catfish			8	2	3						[3]
	Pylodictis olivaris	Flathead catfish											
Atherinopsidae	*Labidesthes sicculus*	Brook silverside	35		11	38 [30]		25[26]	7 [2]			3[6]	[1]
	Menidia beryllina	Silverside	179	29					6			[8]	[8]
Poecilidae	*Gambusia affins*	Mosquitofish	81	3 [2]		6 [1]	9	77 [2]	49 [2]		8	[3]	[14]
Fundulidae	*Fundulus grandis*	Gulf killifish	[7]						[9]				
	Fundulus zebrinus	Plains killifish											
	Fundulus olivaceus	Blackspotted topminnow				[1]						[1]	
Cyprinodontidae	*Cyprinodon rubrofluviatilis*	Red River pupfish											
	Cyprinodon variegatus	Sheepshead minnow							[47]	[7]			

Table 11. ***Continued*** (Sites 1- 11). Abundance of fishes collected at each site during all four seasons. Site information found in Table 10. Numbers listed in brackets are specimens preserved and taken to the Fish Collection at Stephen F. Austin State University. All other individuals were counted and released at the collection site.
Note: *Notropis buccula* and *N. oxyrhynchus* were deposited at the Biodiversity Research and teaching Collection at Texas A&M University in coordination with TPWD and USFWS.

			Sites										
Family	**Scientific Name**	**Common Name**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Moronidae	*Morone chrysops*	White bass											[5]
	Morone saxatilis	Striped bass				[1]							
Centrarchidae	*Lepomis cyanellus*	Green sunfish					1	14	8 [1]	1			
	Lepomis humilis	Orangespot sunfish		28		[3]			23 [4]	14			[1]
	Lepomis macrochirus	Bluegill	26	12	19	13 [5]	6	50	46	3	2	[6]	
	Lepomis megalotis	Longear sunfish	17	3	2	16 [13]	3	31	88 [1]	3	22	[15]	[3]
	Lepomis auritus	Redbreast sunfish		17 [12]									
	Lepomis sp.	Juvenile sunfish										[5]	
	Micropterus punctulatus	Spotted bass		[1]				[1]	11 [3]	6	2	10	6
	Micropterus salmoides	Largemouth bass				6	2	2 [1]	1				[1]
	Micropterus sp.	Juvenile bass							16				
Percidae	*Etheostoma gracile*	Slough darter						32 [8]	2				
	Etheostoma spectabile	Orangethroat darter	47				35		1		4	[4]	
	Percina sciera	Dusky darter				[1]			2	1	2	[1]	
Sciaenidae	*Aplodinotus grunniens*	Freshwater drum											

Table 11. ***Continued*** (sites 12 – 22). Abundance of fishes collected at each site during all four seasons. Site information found in Table 10. Numbers listed in brackets are specimens preserved and taken to the Fish Collection at Stephen F. Austin State University. All other individuals were counted and released at the collection site.
Note: *Notropis buccula* and *N. oxyrhynchus* were deposited at the Biodiversity Research and teaching Collection at Texas A&M University in coordination with TPWD and USFWS.

			Site										
Family	**Scientific Name**	**Common Name**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
Lepisosteidae	*Lepisosteus oculatus*	Spotted gar											
	Lepisosteus osseis	Longnose gar	1			1						1	
Clupeidae	*Dorosoma cepedianum*	Shad					1						
Cyprinidae	*Cyprinella lutrensis*	Red shiner	613 [20]	39 [11]	443 [41]	257 [19]	9 [1]	51	18		542	286 [40]	
	Cyprinella venusta	Blacktail shiner											
	Hybognathus placitus	Plains minnow	8 [46]		201 [6]	11 [11]	48 [55]		36	102 [4]	660 [1]	1752	
	Notropis buccula	Smalleye shiner	208		272 [4]	670	516						
	Notropis oxyrhynchus	Sharpnose shiner	626 [12]	315 [1]	597 [8]	412 [2]	125						
	Notropis potteri	Chub shiner	265 [4]	442 [21]	445 [36]	110 [18]	552 [10]	21		441	156	1958	
	Notropis bairdi	Red River shiner									10 [69]		
	Notropis buchanani	Ghost shiner		67									
	Macrhybopsis hyostoma	Shoal chub	346 [3]	14 [5]	527 [27]	313 [15]	206 [4]	15		6	15	17	
	Pimephales vigilax	Bullhead minnow	9	[7]	1				25		13	92	
	Machrybopsis australis	Prairie chub								9	2		
	Cyprinidae	Juvenile cyprinids			44								
Catostomidae	*Carpoides carpio*	River carpsucker	[1]	[1]								3	
	Ictiobus bubalus	Smallmouth buffalo	3		6								
Ictaluridae	*Ameiurus natalis*	Yellow bullhead						1					
	Ictalurus punctatus	Channel catfish	14 [11]	12 [2]	93 [7]				1				
	Pylodictis olivaris	Flathead catfish			[1]	[1]							
Atherinopsidae	*Labidesthes sicculus*	Brook silverside							1		2	1	
	Menidia beryllina	Silverside	[8]	[5]		[1]		2					

Table 11. ***Continued*** (sites 12 – 22). Abundance of fishes collected at each site during all four seasons. Site information found in Table 10. Numbers listed in brackets are specimens preserved and taken to the Fish Collection at Stephen F. Austin State University. All other individuals were counted and released at the collection site.
Note: *Notropis buccula* and *N. oxyrhynchus* were deposited at the Biodiversity Research and teaching Collection at Texas A&M University in coordination with TPWD and USFWS.

			Sites										
Family	**Scientific Name**	**Common Name**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
Poecilidae	*Gambusia affins*	Mosquitofish	2 [7]	[24]	26 [20]	29 [34]	3 [9]		20	16	8	23	
Fundulidae	*Fundulus grandis*	Gulf killifish											
	Fundulus zebrinus	Plains killifish	[4]	[3]	[4]	247 [77]	4 [33]	154 [30]		165 [79]	3 [55]	2 [25]	[4]
	Fundulus olivaceus	Blackspotted topminnow											
Cyprinodontidae	*Cyprinodon rubrofluviatilis*	Red River pupfish	1 [16]	[1]	[1]	564 [61]	42	[14]		620 [121]	546 [91]	454 [32]	573 [16]
	Cyprinodon variegatus	Sheepshead minnow											
Moronidae	*Morone chrysops*	White bass											
	Morone saxatilis	Striped bass	[3]										
Centrarchidae	*Lepomis cyanellus*	Green sunfish	[1]			[1]				1			
	Lepomis humilis	Orangespot sunfish	1		1		[1]		2	1		3	
	Lepomis macrochirus	Bluegill	1 [5]		1	1	1						
	Lepomis megalotis	Longear sunfish	[2]	[15]					2			7	
	Lepomis auritus	Redbreast sunfish											
	Lepomis sp.	Juvenile sunfish											
	Micropterus punctulatus	Spotted bass		[3]									
	Micropterus salmoides	Largemouth bass	[5]										
	Micropterus sp.	Juvenile bass											
Percidae	*Etheostoma gracile*	Slough darter											
	Etheostoma spectabile	Orangethroat darter											
	Percina sciera	Dusky darter											
Sciaenidae	*Aplodinotus grunniens*	Freshwater drum			1		1						

In comparison with previous surveys (G. Wilde 2009-2017 and Hillis et al. 1980) in the Middle and Upper Brazos River, our collections of the two invasive species at similar localities had lower abundances (Sheepshead: 54 individuals, Gulf killifish: 16 individuals; Fig. 13). Based on the samples provided by G. Wilde, we found Sheepshead minnow was first collected in 2011 in the Brazos River near Possum Kingdom Lake. High abundances of Sheepshead minnow were observed in 2012 and 2013 surveys (Fig. 13) with a decline towards 2014-2015, with no individuals collected in his 2016 or 2017 surveys. On the other hand, the Red River pupfish was collected during all years surveyed by Wilde (Wilde 2015); we also collected Red River pupfish, primarily in sites on the Upper Brazos River, similar sites surveyed by Wilde.

In samples collected by Wilde in 2013 and 2014, we observed a distinct *Cyprinodon* morph that we called as "potential *Cyprinodon* hybrid" as hybridization is supposed to be occurring between Sheepshead minnow and Red River pupfish in the Brazos River (Wilde personal communication, Ayers 2018). For this study, we identified both *Cyprinodon* spp. and *Fundulus* spp. based on the taxonomic key by Hubbs et al. (1991). Therefore, the identification of the "potential *Cyprinodon* hybrid" was solely in observation of color patterns and counts of scales on belly, which suggested an intermediate morph and deemed a putative hybrid. Gene Wilde did not collect the potential hybrids in surveys of 2016 and 2017. Our surveys (2020-2021) did not yield any potential hybrid individuals.

Hillis et al. (1980) reported that Gulf killifish occurs in large numbers in the Brazos River in Hill and Bosque Counties. Our surveys found 7 individuals in McLennan County and 9 in Palo Pinto County across all four seasons (Tables 10 and 11). Cohen et al. (2012) collected Gulf killifish from McLennan to Palo Pinto Counties in the Middle Brazos yield a total of 145. We borrowed 44 individuals of Gulf killifish from the Texas Natural History Collections (TNHC) from these collections made by Cohen et al. (2012) to study ecological aspects. Our surveys in similar sites did not yield similar abundances of Gulf killifish as reported previously for these counties.

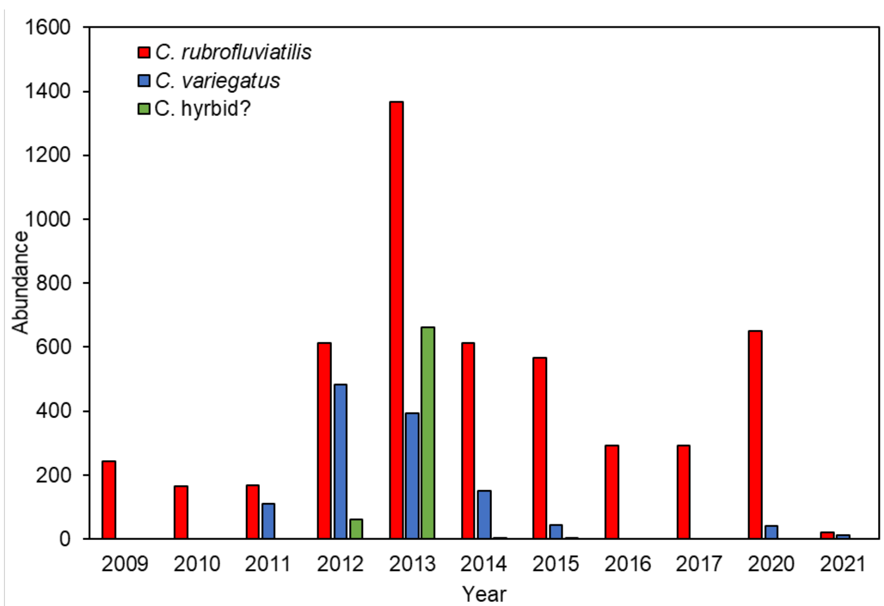

Fig. 13. Abundance of three species of *Cyprinodon* collected in the Brazos River, Texas. Surveys in 2009 to 2017 were conducted by Gene Wilde at Texas Tech University. Surveys in 2020 to 2021 were conducted for the purpose of this project.

Water Quality and Habitat Assessment

A total of 22 sites were surveyed between 2020 and 2021 in both the Brazos River (n=16) and Red River (n=6) basins. These are the same sites surveyed for the invasive cyprinodontid species. At each site, 13 different quantitative environmental variables were measured and average conditions of sites across seasons were calculated. These averages were used in a principal component analysis (PCA) to visualize sites that had similar instream water quality and habitat conditions.

We first performed a PCA analysis of the sites between the two river basins (Fig. 14). Clearly, it shows a separation on the sites based on water conditions, water depth and current (Fig. 14). The PCA resulted in two main axes that explained 49.25% of the environmental variation (PC1=33.17%, PC2=16.80%). The PCA 1 showed a gradient separating site from Brazos and Red River (Fig.14). Sites in the Upper Red River were shallower, with higher conductivity, higher total dissolved solids and more turbid (especially at Wichita River SH-25). While sites in the Brazos River had deeper water and lower salinity and conductivity (especially those around the Middle Brazos), larger wetted widths (Fig. 14). The PC 2 axis showed a gradient separating those sites with faster current, more basic pH, and higher ammonia ($NH+_4$) concentration from those sites containing higher nitrate (NH_{3-}) concentration, and higher salinity. With few exceptions (Site 18, Wichita River SH-25), there was a general pattern in which sites locates in the Upper Brazos River and the Red River were more similar in their environmental conditions. This makes sense due to the geographic location and geological formation of these two sections of river. River sites in this region are relatively shallow, sandy bottomed, and murky water.
A second PCA analysis was conducted in sites of the Middle and Upper Brazos River (Fig. 15).

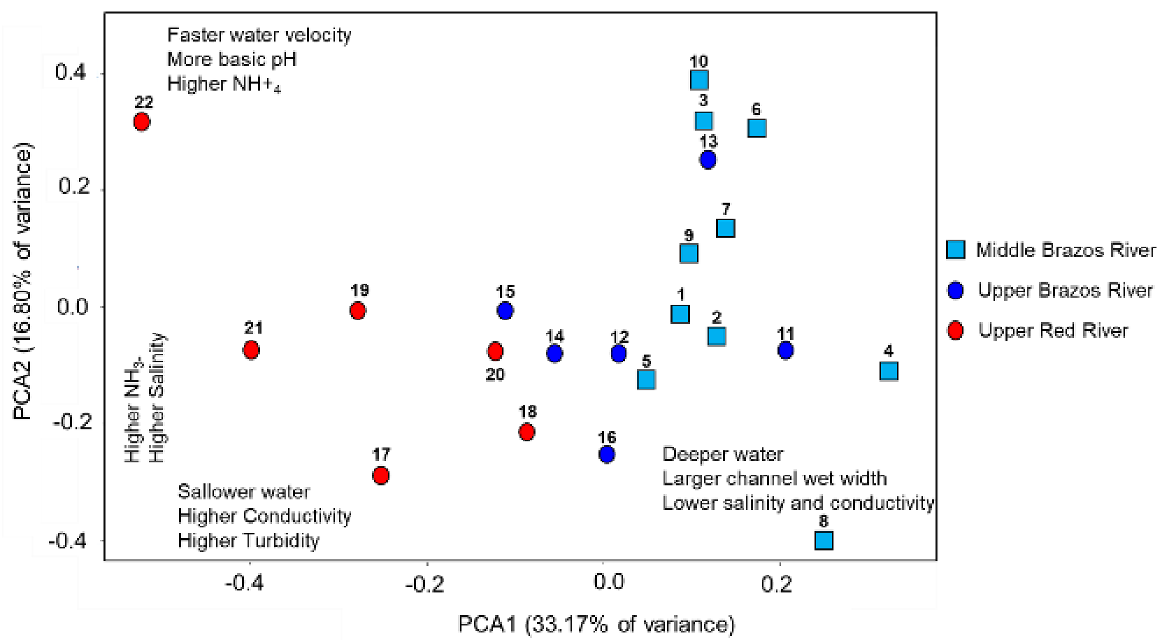

Fig. 14. Principal component analysis conducted on the averages of 14 environmental variables of sites along the Upper Brazos River (n=6), Middle Brazos River (n=10), and Upper Red River (n=6). Numbers indicate sites sampled (see Table 9 for site details).

The PCA showed a separation of the sites primarily based on the water conditions (Fig. 15). For example, the PCA 1 (explained 37.10% of the variation) showed a gradient separating those sites from Middle Brazos which contained lower salinity and conductivity from those sites in the Upper Brazos with higher salinity and conductivity (Fig. 15). Sites in the Upper Brazos also appeared to have higher concentrations of nitrates (NH_{3-}) and ammonium (NH_{+4}). The PCA 2 (explained 17.93% of the variance) also suggested a gradient of the sites based on salinity and conductivity, with sites in the Middle Brazos being relatively deeper and with larger wetted widths than sites in the Upper Brazos. These results agree with previous studies by Mayes et al. (2019) and Ostrand & Wilde (2002), who suggested that waters in the upper Brazos River have higher salinity and conductivity. Visually, sites located in the Upper Brazos River and Middle Brazos River are generally different in term of habitat conditions (Figs. 16, 17). For example, sites in the Middle Brazos are deeper, with cobble and more gravel substrates, and more clear water (Fig. 16), while sites located in the Upper Brazos River are shallower, with sandy substrates, and having more turbid water (Fig. 17). Also, sites in the Upper Brazos were more similar those in the Upper Red River (Fig. 18).

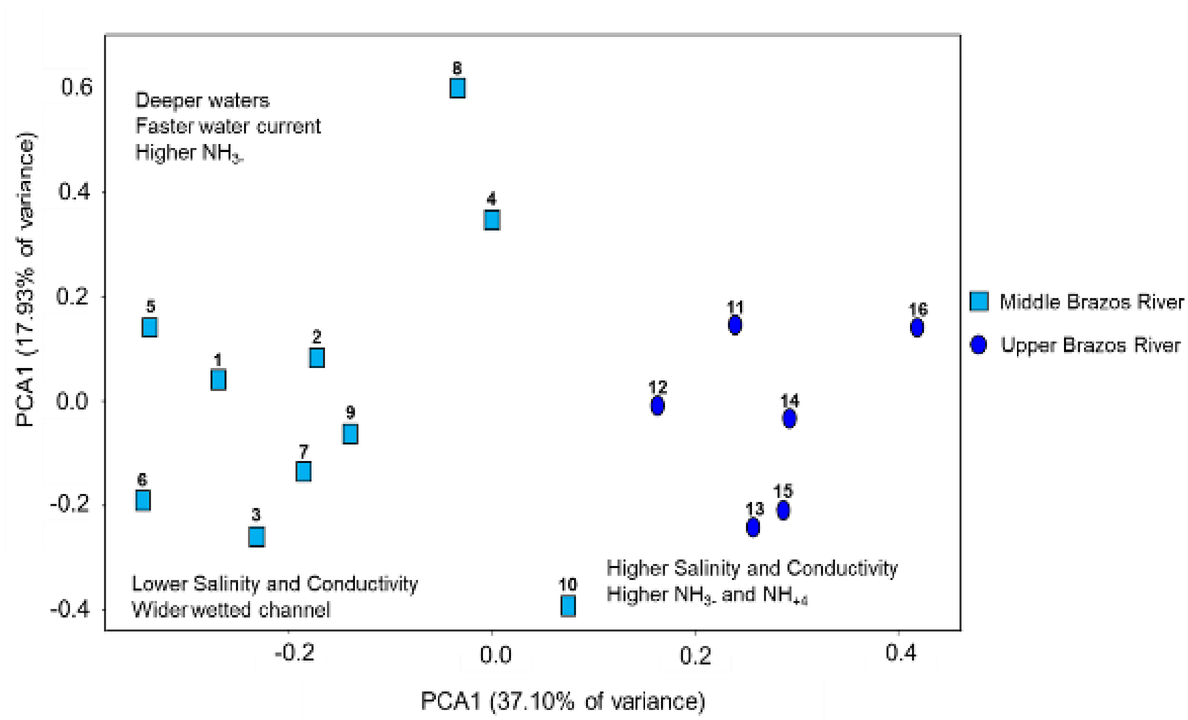

Fig. 15. Principal component analysis (PCA) conducted on 14 environmental variables of sites along the Upper Brazos (n=6) and Middle Brazos (n=10) basins. Numbers indicate sites sampled (see Table 9 for details).

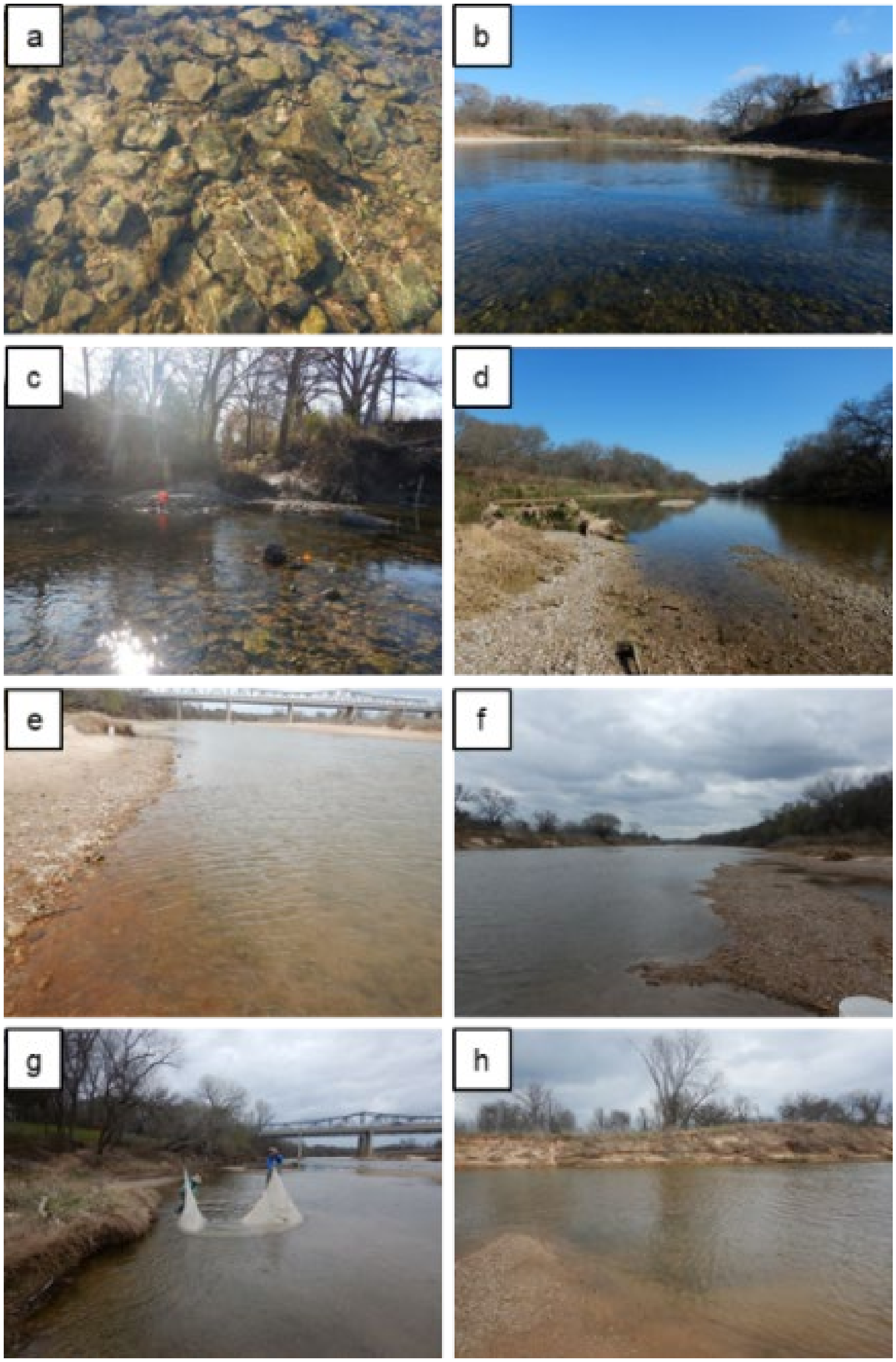

Fig. 16. Photos showing representative habitats of sites sampled in the Middle Brazos River, Texas. Photos **a-d** were taken at Site 1, McLennan County, TX on December 14, 2020. Photos **e-h** were taken at Site 7, Palo Pinto County, TX on March 12, 2021.

Fig. 17. Habitats sampled in the Upper Brazos River, Texas. Photos **a-d** were taken at Site 12, Young County, TX on May 14, 2021. Photos **e-h** were taken at Site 14, Throckmorton County on May 14, 2021.

Fig. 18. Photos showing representative habitats of sites sampled in the Upper Red River, Texas. Photos **a-d** were taken at Site 20, Wilbarger County, TX on October 30,2020. Photos **e-h** were taken at Site 21, Hardeman County, on the Texas-Oklahoma boarder on May 13, 2021.

Morphological diversity of focal Cyprinodontiformes

A total of 750 specimens (*C.* hybrid? n=50, Red River pupfish n=405, Sheepshead minnow n=107, Plains killifish n=168, and Gulf killifish n=60) were measured for 25 different morphological traits related to feeding ecology and habitat use. For *Cyprinodon* species, we measured specimens that were collected during our surveys in 2020-2021 in both the Brazos and Red Rivers (Red River pupfish n=211, Sheepshead minnow n=34), but also specimens that were collected by G. Wilde (2009-2015) (*C.* hybrid? n=50, Red River pupfish n=194, Sheepshead minnow n=73). For *Fundulus* species, we measured those specimens that were collected during the 2020-2021 survey (Plains killifish n=168, Gulf killifish n=16) and in addition, we measured 44 specimens of Gulf killifish that were collected by Cohen et al. (2012).

All morphological traits, except for mouth position, were then transformed into ratios following protocols outlined by Winemiller (1991). Morphological trait ratios were then log transformed and used in a principal component analysis (PCA) to visualize functional trait space occupied by each species and potential functional niche overlap. Fig. 19 shows a PCA analysis of the two *Cyprinodon* species and the "potential *Cyprinodon* hybrid". The PCA resulted in two main axes that explained 37.35% of the morphological variation (PC1=23.21%, PC2=14.14%) and shows a large degree of overlap in functional trait space among the three *Cyprinodon* morphs (Fig. 19). The PCA 1 shows a gradient separating those individuals with longer pectoral fins, deeper peduncles, and shorter heads (low and negative scores) from individuals having longer heads and broader body (Fig. 19). The latter was the case for some individuals of Sheepshead minnow. For PCA 2, on the other hand, the gradient was mostly associated with traits related to trophic ecology such as snout length, body width, gape width, snout protrusion. Individuals with high (positive) scores on PCA 2 had larger gape widths, longer snout protrusion, and longer peduncles. This was the case for both Red River pupfish and the potential *Cyprinodon "*hybrid" individuals (Fig. 19), while that individuals with low (negative) scores in PCA 2 had wider peduncles, longer snouts, and narrower bodies, and correlates with individuals of Sheepshead minnow (Fig. 19).

While there were differences in morphological trait space observed among all three *Cyprinodon* species, there was still a large degree of overlap (Fig. 19). Sheepshead minnow is shown to have a larger functional trait space, with some individuals having longer heads, narrower bodies, and longer snouts. Some individuals of Sheepshead completely overlap with Red River pupfish and *Cyprinodon "*hybrid" individuals. The Red River pupfish derived from an ancestor similar to Sheepshead, so morphological similarities are expected (Miller & Echelle 1975, Minckley 1980). This overlap in functional traits associated with feeding ecology and habitat use could have consequences on the fitness of Red River pupfish and other closely related inland *Cyprinodon* species if Sheepshead continues to invade inland Texas streams.

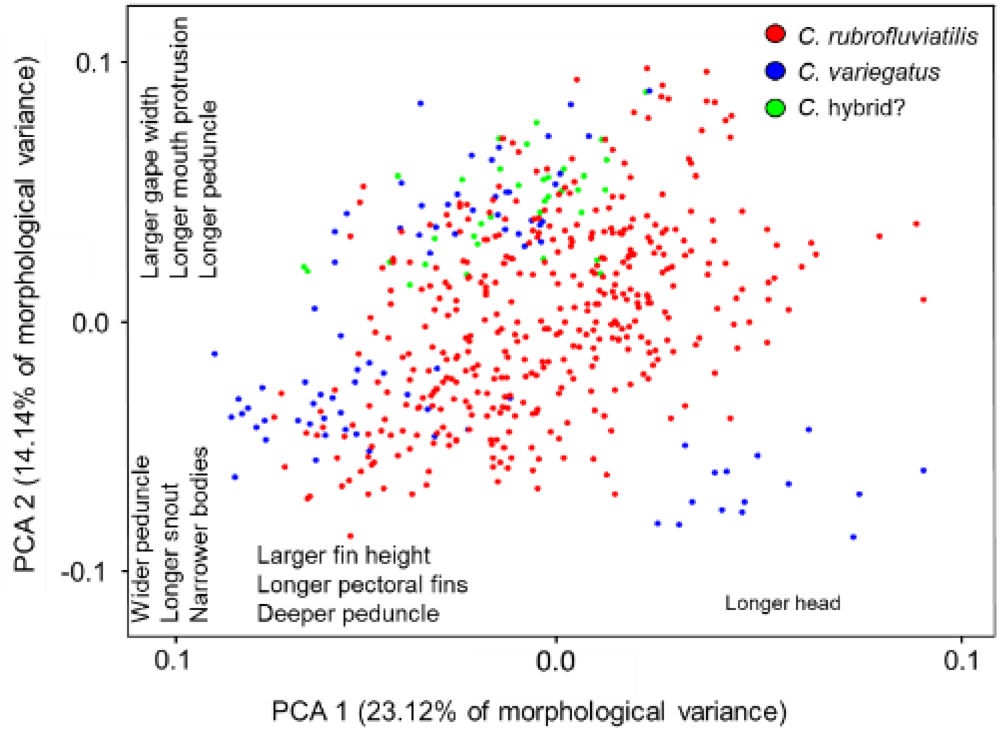

Fig. 19. Principal component analysis (PCA) conducted on 25 morphological traits of Red River pupfish (*C. rubrofluviatilis*), Sheepshead minnow (*C. variegatus*) and potential *Cyprinodon* "hybrids", collected during both Wilde (2009-2017) survey and contemporary 2020-2021 surveys in the Brazos and Red River basins, Texas.

Fig. 20 shows the morphological analysis (PCA analysis) of the two *Fundulus* species. Overall, we did not observe significant overlap on their functional trait space. The PCA resulted in two main axes that explained 33.97% of the morphological variation (PC1=24.51%, PC2=9.46%). Positive scores on PCA 1 were associated with individuals having shorter anal, dorsal, and caudal fins (e.g., Plains killifish), while negative scores were associated with larger individuals having deeper peduncles, larger head height, and longer pelvic fins (e.g., Gulf killifish, Fig. 20). PCA 2, on the other hand, shows a gradient separating those individuals with larger and deeper bodies (positive scores Fig. 20) from individuals with smaller bodies, smaller eyes and snout (e.g., Plains killifish). Unlike the *Cyprinodon* species, we see a clear divide between the functional trait space occupied by each species with little overlap in functional trait space.

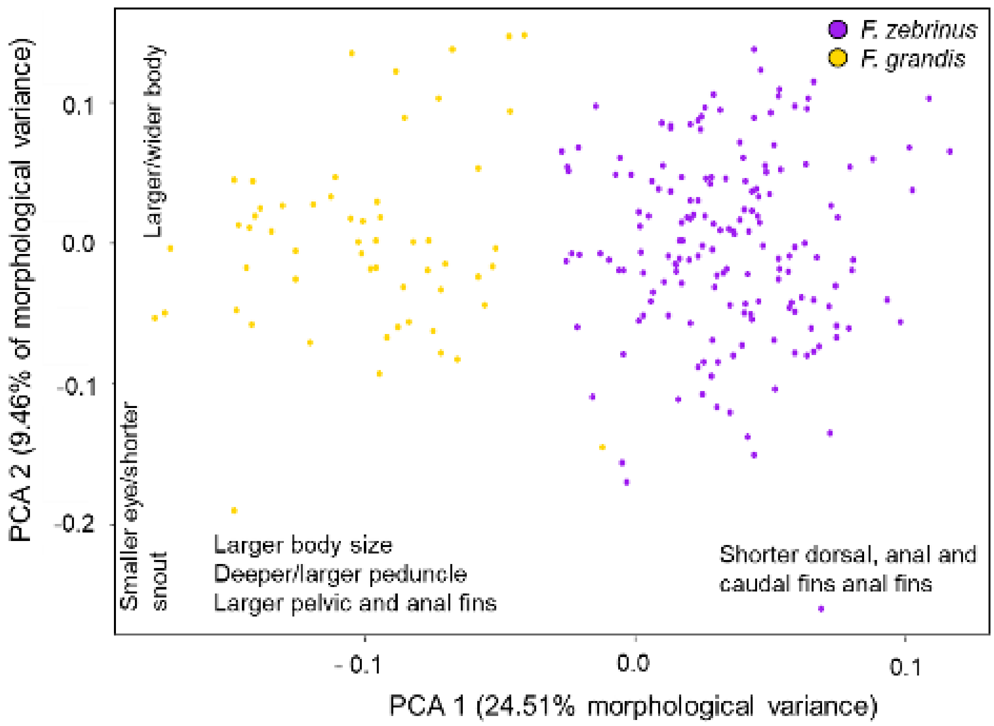

Fig. 20. Principal component analysis (PCA) conducted on 25 morphological traits of Plains killifish (*F. zebrinus)* and Gulf killifish (*F. grandis)* collected during our contemporary 2020-2021 surveys and from specimens collected by Cohen et al. (2012) in the Brazos River.

Feeding Ecology of Focal Species

Cyprinodon species

Stomach contents analysis were conducted in specimens collected from our surveys (2020-2021) and those collected by Wilde (2009-2017). In total, we examined 489 individuals containing Red River pupfish (n=318), Sheepshead minnow (n=131), and *Cyprinodon* "hybrid" (n=40).

Prey items found in the stomachs were identified to the lowest taxonomic level possible and then placed into major groups to better understand diet patterns of each species. The percentage of prey items consumed by volume was calculated. Thirty-five (35) different prey items were identified and placed in the following categories: detritus, algae, plant material, sand/gravel, microplastics, Diptera, Trichoptera, Odonata, Hymenoptera, Coleoptera, Hemiptera, unidentified insect parts, Nematoda, Annelida, Ostracoda, Gastropoda, fish parts, microorganisms.

Overall, the three *Cyprinodon* morphs appeared to consume similar food items (Fig. 21). The most abundant (>1.0%) item ingested by volume by the three morphs was detritus (Fig. 21), with Sheepshead minnow showing a higher consumption (70.4%) compared to Red River pupfish (54.5%) and the potential *Cyprinodon* "hybrids" (52.8%). Ingestion of sand/gravel was also high in Red River pupfish and the potential *Cyprinodon* "hybrid". The three morphs consumed aquatic invertebrates within the order Diptera being the most consumed. Although Sheepshead minnow appeared to consume higher percentage of dipteran (10.1%) compared to its congener Red River pupfish (4.2%) and the potential “hybrid” (4.8%) (Fig. 21). Sheepshead minnow also consumed more ostracods (1.4%) that the other two *Cyprinodon*. Other unidentified food items were present in the stomach contents for each species, but they made up <1.0% of stomach contents by volume.

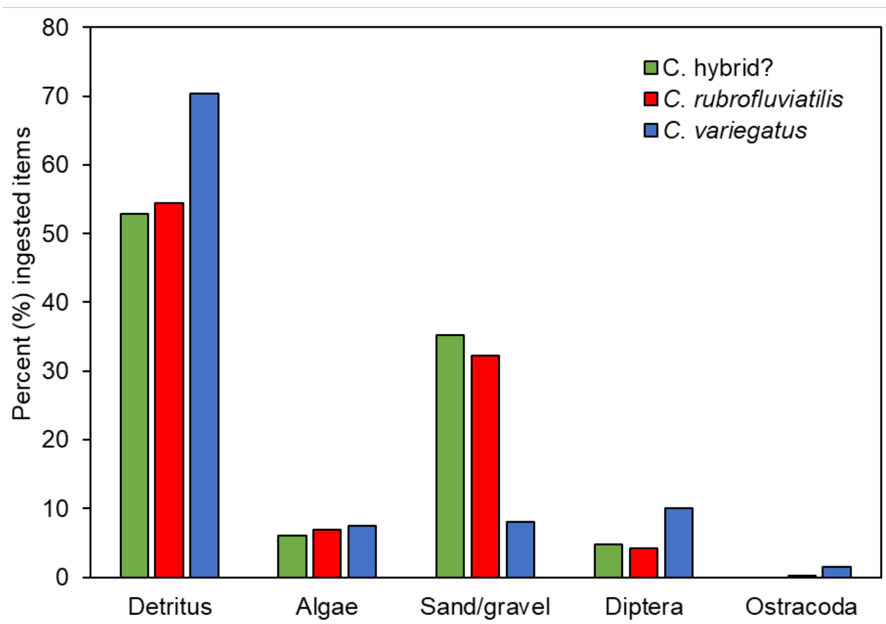

Fig. 21. Percentage (%) of food items identified in stomach contents analysis for each *Cyprinodon* species. Only food items consisting of >1.0% were included.

Overall, all three *Cyprinodon* species showed similar patterns in food items observed in stomach contents. The Red River pupfish and *Cyprinodon* “hybrid” shared almost identical food preferences based on their volumetric food items. Sheepshead minnow also showed similar patterns in food items consumed, but stomach contents contained ostracods, higher portions of detritus and dipteran, and lower portions of sand/gravel.

Prey items within the order Diptera were identified down to family when possible. Fig. 22 shows the list of dipterans consumed by the three *Cyprinodon* morphs. For example, the three morphs appeared to consume Chironomidae in high proportions (Red River pupfish with 92.0%, Sheepshead 86.6% and *Cyprinodon* “hybrid” with 100%, Fig. 22). Red River pupfish also consumed other invertebrate taxa in low frequency (<4.0%) and included items in the families Dolichopodidae, Dixidae, Ceratopogonidae, and Tabanidae. Sheepshead minnow on the other hand, was a more generalist feeder than Red River pupfish, consuming a wider variety of insect larvae including items in the families Thaumaleidae, Dolichopodidae, Tanyderidae, Ceratopogonidae, Tipulidae, and Empididae (Fig. 22).

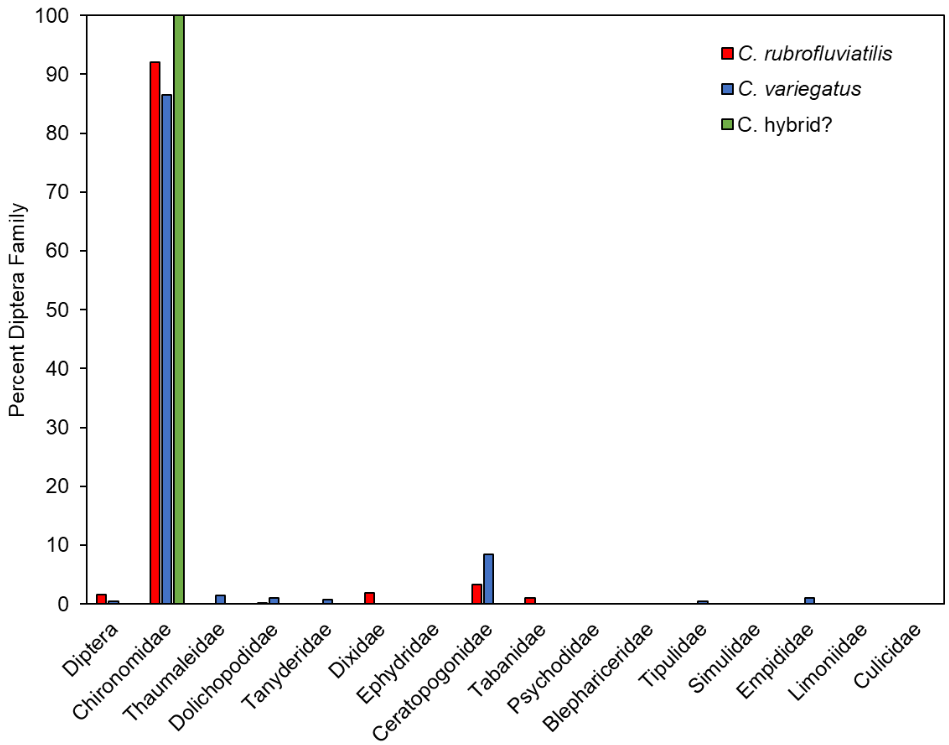

Fig. 22. Percentage (%) of families within the order Diptera consumed by Red River pupfish (*C. rubrofluviatilis*), Sheepshead minnow (*C. variegatus*) and the potential *Cyprinodon* "hybrid". Items that were not able to be identified to family were left under the

Our findings on dietary habits of Red River pupfish and Sheepshead minnow agree with previous studies (Echelle 1973, Miller & Robison 2004). The Red River pupfish has been shown to be an intensely active bottom-feeding omnivore that feeds mostly on midge (Diptera) larvae (Echelle 1973, Miller & Robinson 2004). It has been reported to "nip" and "plow" at the bottom to push sediment aside to reveal insect larvae and other food items (Minckley & Arnold 1969, Echelle et al. 1972). This feeding behavior suggests that individuals ingest large amounts of substrate such as sand and gravel, as observed in our stomach contents analysis. Sheepshead minnow is also shown to be an omnivorous fish, and is reported to feed largely on organic detritus, algae, microcrustaceans, and dipteran larvae (Harrington & Harrington 1961). Sheepshead minnow has been shown to display "plowing" behaviors as it picks substrate for food items, similar to Red River pupfish (Foster 1967). We did observe a lower percentage of sand and gravel consumed by Sheepshead minnow and a higher percentage of organic detritus consumed. Both detritus and sand are most likely accidentally ingested when individuals pick at the bottom in search of insect larvae. The differences observed between species suggests that they may be occurring at locations along the streams with different substrates. Red River pupfish is reported to have a strong preference for sandy bottoms, while Sheepshead minnow seems to be less discriminating in that regard (Minckley et al. 1991).

Our data suggest that Red River pupfish and the potential *Cyprinodon* "hybrid" have almost identical patterns in diet based on stomach contents analysis. While the diet of Sheepshead minnow does differ slightly, there is still a large degree of overlap with the native species. This overlap in dietary niche indicates that, if occurring together, Red River pupfish potentially could compete for food resources with both the invasive and potential “hybrid” species. This could result in devasting impacts on the fitness of the native species if Sheepshead minnow and the potential *Cyprinodon* “hybrid” populations continue to persist in the Brazos River.

Fundulus species

A total of 276 killifish were examined for stomach contents analysis, which were comprised by 216 Plains killifish (*F. zebrinus)* and 60 Gulf killifish (*F. grandis)* specimens. For the Gulf killifish, 16 individuals were analyzed from our surveys of 2020-2021 and 44 from collection made by Cohen et al. (2012). In total, seventy (70) different prey items were identified in the stomach contents of the two species of killifish and placed into the following categories: detritus, algae, unidentified plant material, sand/gravel, microplastics, Diptera, Trichoptera, Odonata, Hymenoptera, Coleoptera, Hemiptera, Megaloptera, Lepidoptera, Plecoptera, Ephemeroptera, unidentified insect parts, Nematoda, Annelida, Crustacea, Gastropoda, and fish parts.

Overall, the two species of killifish had a more diverse diet that *Cyprinodon* spp*.* The most abundant (>1.0%) items ingested by the two killifish was detritus (Fig. 23), although the percentage of consumption varied, with the Plains killifish having a higher percentage of detritus ingestion compared to the Gulf killifish (64.2% to 49.4%, respectively). Both species of killifish consumed similar types of invertebrate items. However, it is clear that the Gulf killifish was consuming higher proportions of odonates, fish parts, gastropod, and hemipteran compared to Plains killifish (Fig. 23). Other food items (e.g., plastic filaments) were present in the stomach contents for each species but they made up <1.0% of stomach contents by volume.

While similarities in the diet of the two killifish species was observed. There are also differences in their dietary patterns, with Gulf killifish consuming a larger portion and greater variety of aquatic macroinvertebrates and fish compared to the Plains killifish. Stomach contents for Plains killifish showed mostly detritus and aquatic insects abundant in three orders (Diptera, Trichoptera, Hemiptera). Gulf killifish on the other hand, showed less detritus, aquatic insects abundant in four orders (Diptera, Odonata, Hemiptera, Ephemeroptera), and three other phyla and subphyla (Nematoda, Crustacea, Gastropoda) (Fig. 23). Gulf killifish also showed more piscivory compared to Plains killifish, suggesting that, if occurring together, there could be negative impacts (potential for predation) on Plains killifish, but also for other native species (such as cyprinid larvae) in invaded habitats. Similar to Sheepshead minnow, the Gulf killifish appears to be a more generalist feeder than its native congener.

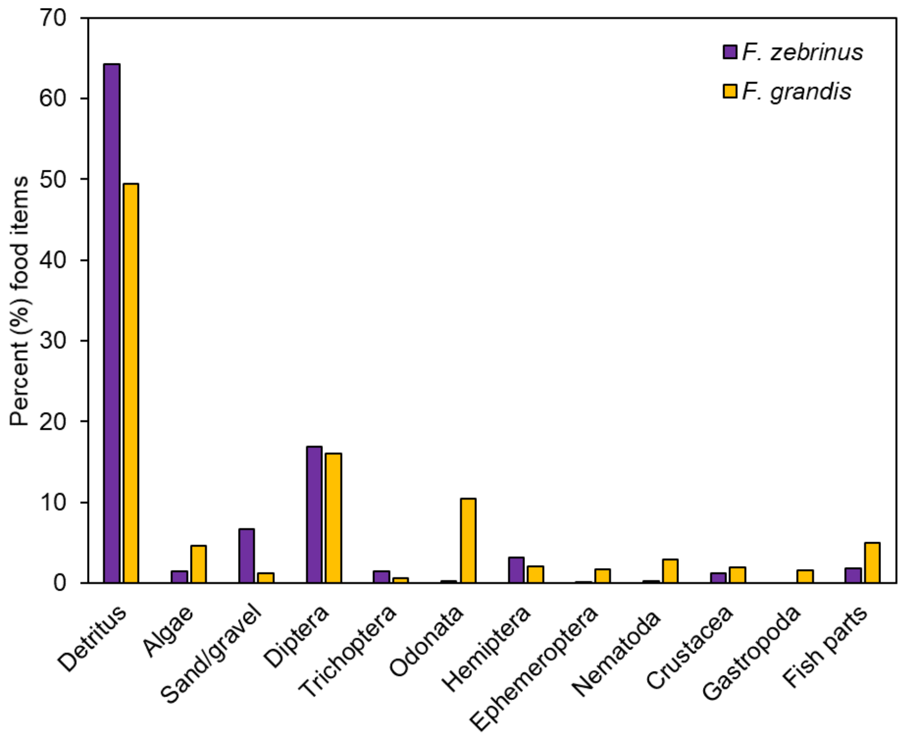

Fig 23. Percentage (%) of food items identified in stomach contents analysis of Plain killifish (*F. zebrinus)* and Gulf killifish (*F. grandis)*. Only food items consisting of >1.0% included in the analysis.

Previous studies have examined feeding habits of both Plains killifish and Gulf killifish. The Plains killifish has been shown to have carnivorous habits, consuming mostly midge (dipteran) larvae and other aquatic invertebrates (Minckley & Klaassen 1969a, Echelle et al. 1972). Similarly, we observed that a large portion of the diet of the Plains killifish consisted of dipteran larvae (16.9%). Both studies above also documented a considerable amount of miscellaneous detritus and sand in the stomach contents of Plains killifish, in agreement with our findings. Echelle et al. (1972) suggests that this material is largely undigested and was likely ingested during times of low visibility in search of other prey items. At sites where Plains killifish was abundant, such as in the Pease and Red rivers, the water often was more turbid. This could have impacted to fish's ability to visually located food and help explain large portions of detritus observed in stomach contents.

Vaughan et al. (2016) investigated the feeding ecology of the Gulf killifish in the Pecos River, another inland location where it had been introduced. They report that this species consumed mostly fish (77.8% by volume) and contained a relatively low portion of aquatic insects (2.9% by volume) and detritus (8.2% by volume). We observed that the fish item made up only 4.9% of the diet of Gulf killifish, with aquatic insect across all orders making up 32.5% and detritus 49.4% of their diet. Vaughan et al. (2016) suggest that the Gulf killifish may be filling a unique niche in the highly-salinity reaches of the Pecos River where other larger piscivorous fish are absent. Although in low abundance, we did capture large piscivorous fish in the Middle Brazos River such as gar (*Lepisosteus* sp.) and bass (*Micropterus* sp.) species in same locations of the

Gulf killifish, however, there was high segregation with bass and gars occupying deeper water than the Gulf killifish. The presence of large piscivorous in the Brazos and not in the Pecos could explain the difference in food items in stomach contents found in Vaughan et al. (2016) study and ours, hence this is just an inference about the results. All our Cyprinodontid species showed a substantial consumption of detritus and sand/gravel. As previously discussed, such items could mostly likely accidentally ingested when searching for small insect larvae located in the substrate.

To further investigate feeding trends and better understand what food items are not only being ingested but also assimilated into consumers tissues, we performed a stable isotope analysis (SIA) on tissue samples of all four Cyprinodontiformes. Tissue samples were analyzed for $\delta^{13}C$ and $\delta^{15}N$ signature, where carbon is assumed to reflect how material from various production sources is assimilated into consumer tissue and nitrogen reflects vertical trophic position of consumer (Montaña et al. 2020, Fig. 24). In total, 550 fish were processed and sent to the Analytical Chemistry Laboratory, Institute of Ecology, at the University of Georgia for stable isotope analysis (potential *Cyprinodon "* hybrid" n=39, Red River pupfish n= 197, Sheepshead minnow n=102, Plains killifish n=152, Gulf killifish n=60). We used tissue samples from specimens collected by G. Wilde, Cohen et al. (2012) and from our own surveys. In addition, we collected 82 samples of primary producers along sites in the Brazos and Red Rivers.

Due to delays from Stable Isotope Analysis at the University of Georgia, which are associated with COVI-19 situation, we are still missing isotope reports for samples submitted in July 2021. This includes 288 fish tissues of *Cyprinodon* and *Fundulus* species and 43 samples of basal resources, which were collected in our late spring 2021 surveys.

Results based on 37 samples of basal resources including benthic algae, sediments, leaf litter, aquatic grasses and aquatic macrophytes suggest the potential for aquatic macrophytes (mean [±SD] $\delta^{13}C$: -26.7‰ [±0.50]). Carbon ($\delta^{13}C$) derived from benthic algae and leaf litter (perhaps from allochthonous sources) were very depleted in $\delta^{13}C$ (-29.07‰ and -29.7‰, respectively), while sediments and aquatic grasses were more enriched in $\delta^{13}C$ (-8.33‰ and -14.41‰, respectively).

A biplots of $\delta^{13}C$ and $\delta^{15}N$ values was made to depict the overlap in the isotopic niche space occupied for the *Cyprinodon* species (Fig. 24) and the two *Fundulus* species (Fig. 25). First, all three *Cyprinodon* species showed similar patterns in $\delta^{13}C$ and $\delta^{15}N$ values fixed in fish tissues. However, there were two clusters of Sheepshead minnow that varied in $\delta^{13}C$ and $\delta^{15}N$ signatures (Figs. 24 and 26). These clusters came from samples that were collected from two locations, Graham and Elbert in Texas, where the invasive Sheepshead minnow, native Red River pupfish, and potential *Cyprinodon* "hybrid" were found together (See Fig. 9 for location details). A group of 12 Sheepshead minnow collected around Graham sites were more enriched in $\delta^{15}N$ (14.8‰ and 18.2‰) as all other individuals, but more depleted in $\delta^{13}C$ (-19.1‰ and - 17.4‰, 26a). Another group of 10 Sheepshead minnow also collected at sites in Graham were enriched in carbon ($\delta^{13}C$-19‰ and -15‰) (Fig. 26), as the majority of other individuals, but were more enriched in nitrogen ($\delta^{15}N$: 15‰ and 19‰). At the Elbert site, the three *Cyprinodon* morphs showed high overlap in the isotopic niche ratios (Fig. 27). We also performed a regression analysis to see if there was a correlation between nitrogen signatures and standard length, but no strong correlation was found (y=0.74x-9.33, R^2=0.30). We did find that the individuals with different isotopic signatures were collected a one site, Graham, Texas, in 2013 and 2014. Since we do not have information on the water quality/habitat at the survey sites and time neither information in production sources, we speculate that variation in the isotopic signature may have been due to environmental conditions in site.

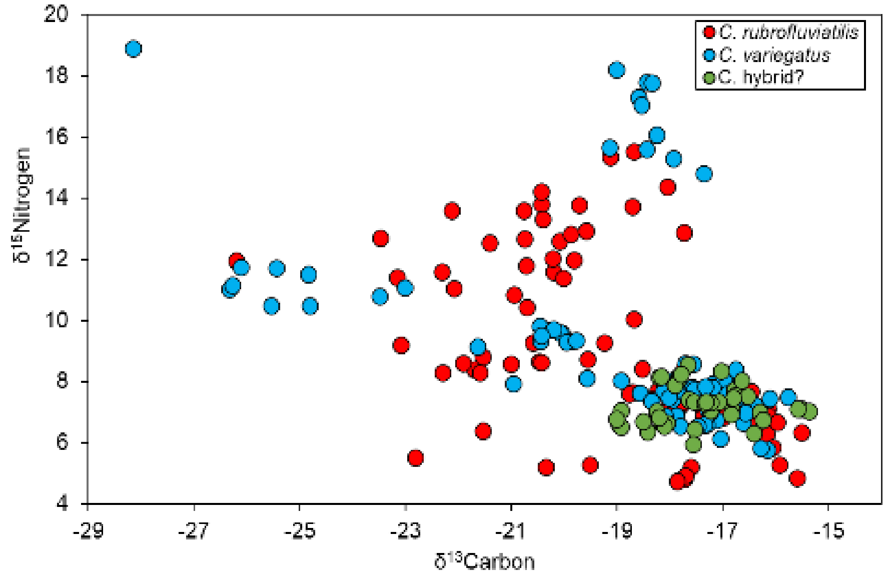

Fig. 24. Biplot of $\delta^{13}C$ and $\delta^{15}N$ signatures fixed in muscle tissues of individual *Cyprinodon* species.

The two species of *Fundulus* did not show overlap in their isotopic niche space (Fig. 25). The Gulf killifish was more depleted in $\delta^{13}C$ (-27‰ and -25‰) compared to Plains killifish (-23‰ and -17‰). Gulf killifish were also more enriched in δ15N than most Plains killifish. This suggest that Gulf killifish may be utilizing a different carbon source, and potentially be feeding higher in the food chain than its native congener.

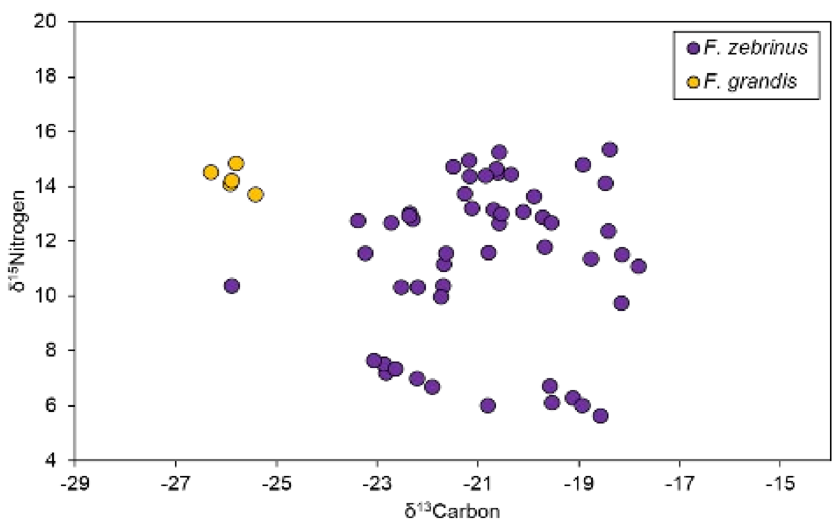

Fig. 25. Biplot of $\delta^{13}C$ and $\delta^{15}N$ signatures fixed in muscle tissues of two species of *Fundulus*.

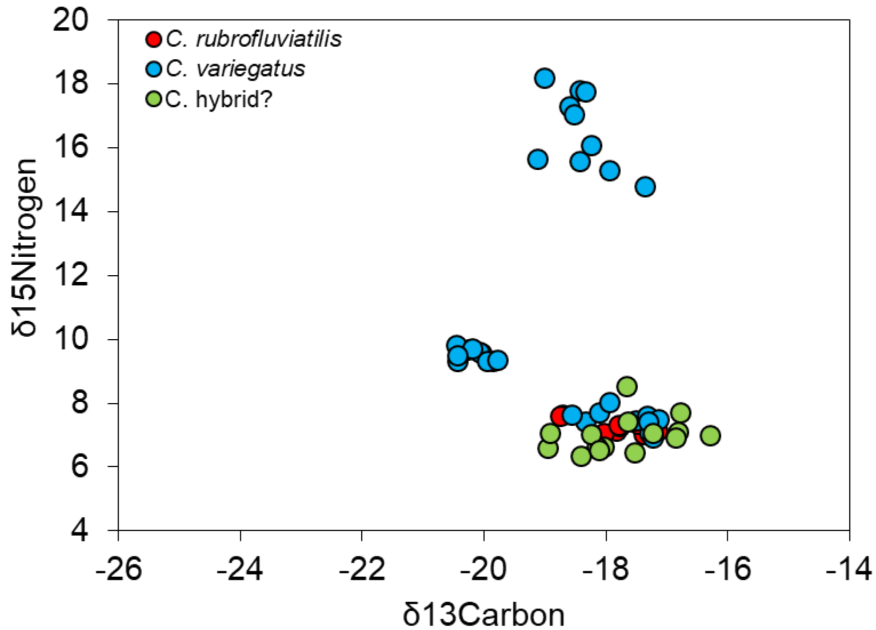

Fig. 26. Biplot of $\delta^{13}C$ and $\delta^{15}N$ signatures fixed in muscle tissues of the three *Cyprinodon* morphs occurring in Brazos River at Graham sites. Specimens were collected by Gene Wilde between 2012 and 2014.

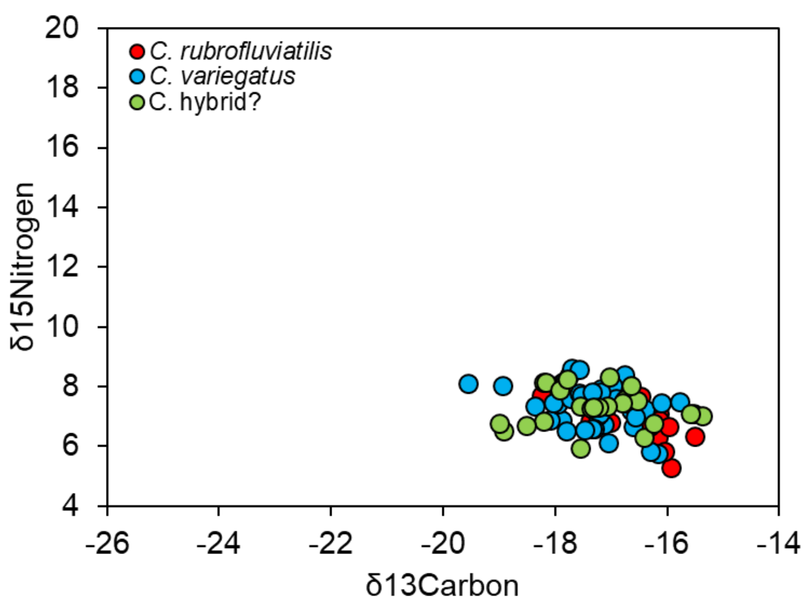

Fig. 27. Biplot of $\delta^{13}C$ and $\delta^{15}N$ signatures fixed in muscle tissues of the three *Cyprinodon* morphs occurring in Brazos River at Elbert site. Specimens were collected by Gene Wilde between 2012 and 2014.

Task 4. Landscape modelling for invasive coastal fishes introduced to inland waters: a case with Sheepshead minnow and Gulf killifish

4.1 Methods and procedures

Riverscape data

We used the medium resolution National Hydrography Dataset (i.e., NHDplus version 2) as the source for stream locations and riverscape attributes (McKay et al. 2012). The grain size for our modelling was inter-confluence stream segments for which multiple riverscape attributes are developed. These attributes included stream fragmentation metrics developed by Cooper and Infante (2017) and anthropogenic alterations developed by Herreman et al. (2017). We used the NHDplusV1 to NHDplusV2 crosswalk to ensure attributes assigned to version 1 stream segments were appropriately assigned to version 2 stream segments. We began with the full suite of metric derived by Cooper and Infante (2017) and Herreman et al. (2017) and removed variables with absolute correlation coefficients of 0.70 to avoid multi-collinearity. The subset of attributes retained for analysis are given in Table 12. Prior to analysis, all variables were z-score transformed to a mean of zero and standard deviation of one to better approximate normal distributions.

Fish occurrence data

We obtained georeferenced occurrence records for Sheepshead minnow (*C. variegatus*) and Gulf killifish (*F. grandis*) from the fishes of Texas database (Fig. 28). We filtered collections to remove those that included only one or two species from the same location on the same date to remove single species records and retain fish community samples (Perkin & Bonner 2011). We then coded the presence of either *C. variegatus* or *F. grandis* as “1” and the absence of respective species using “0”. Next, we joined attributes from the NHD inter-confluence stream segments to fish collections that occurred on each of the segments using ArcGIS. Fish collections that were georeferenced to locations >0.25 km from target stream segments were excluded from analysis.

Ecological niche modelling

We used random forest models to generate an ecological niche model (ENM) for each invasive separately. Because there were fewer presence data points than absence data points, we used the synthetic minority oversampling technique (SMOTE) described by Chawla et al. (2002) to generate a balanced dataset composed of equal numbers of presence and absence data. We then used the ‘randomforest’ function from the ‘randomForest’ package in R to fit models. We assessed variable importance using mean decrease in Gini, which illustrates the loss of classification accuracy when individual variables were excluded form models. A large mean decrease in Gini represents an important variable in terms of creating accurate classifications of presence or absence. We also used the ‘predict’ function in R to generate predictions for the probability of occurrence for each species at 3,821 stream segments distributed across the Brazos and Red River basins.

Model validation data

We collected field data from 22 sites during 2020 and 2021 and used these data to assess model performance on an independent dataset. We used the ‘confusionMatrix’ function to generate estimate model accuracy, which ranges 0-1 (0 = predictions wrong for all 22 sites; 1 = predictions correct for all 22 sites). We report the estimated accuracy values as well as 95% confidence intervals for each model to assess performance on newly collected data. Because there were few presence records in the validation dataset, we used the SMOTE routine to balance the validation dataset prior to assessing model performance.

Table 12. Riverscape variables, definitions, and sources used in ecological niche modelling.

Variable	Definition	Source
SLOPE	slope of stream segment	McKay et al. (2012)
UM2D	distance to upstream dam	Cooper et al. (2017)
DM2D	distance to downstream dam	Cooper et al. (2017)
L_POPDENS	human population in local network	Herreman et al. (2017)
L_ROAD_CR	road crossings in local network	Herreman et al. (2017)
L_URBANL	low intensity urban land use in local network	Herreman et al. (2017)
L_SLOPE	average stream slope in local network	Herreman et al. (2017)
L_ELEVATION	average elevation in local network	Herreman et al. (2017)
L_PASTURE	percent pasture land use in local network	Herreman et al. (2017)
L_CROP	percent crop land use in local network	Herreman et al. (2017)
N_POPDENS	human population density in upstream network	Herreman et al. (2017)
N_ROAD_CR	number of road crossings in upstream network	Herreman et al. (2017)
N_PHOS_YIE	estimated phosphorous yield in upstream network	Herreman et al. (2017)
N_SED_YIEL	estimated sediment yield in upstream network	Herreman et al. (2017)
N_URBANL	low intensity urban land use in upstream network	Herreman et al. (2017)
N_URBANH	high intensity urban land use in upstream network	Herreman et al. (2017)
N_PASTURE	percent pasture land use in upstream network	Herreman et al. (2017)
N_CROP	percent crop land use in upstream network	Herreman et al. (2017)
N_GWINDEX	percent of base flow contribution by groundwater	Herreman et al. (2017)
N_PRECIP	average precipitation in upstream network	Herreman et al. (2017)
N_AREASQKM	upstream drainage area	Herreman et al. (2017)
N_TOTAL_WD	total upstream water withdrawals	Herreman et al. (2017)

4.2 Results and discussion

The modelling dataset included 64 locations (33 present, 31 absent) for Sheepshead minnow and 84 locations (41 present, 43 absent) for Gulf killifish. The raw out of bag (OOB) error rate for the Sheepshead minnow model was 7.81%, suggesting high classification accuracy. Twenty-nine of the absence records and 30 of the presence records were correctly predicted by the model. When validated with the independent dataset, the accuracy of the model was 0.92 (95% CI = 0.62-0.99). The raw OOB error rate for the Gulf killifish model was 5.95, suggesting high classification accuracy. Forty of the absence records and 39 of the presence records were correctly predicted by the model. When validated on the independent dataset, the accuracy of the model was 0.83 (0.52-0.98).

The most influential variables in the Sheepshead minnow model were related to land cover and land use, groundwater input, stream size and slope, and human population density. The most influential variable was the percent of the upstream network covered by cultivated crops, followed closely by the percent of base flow contributed by groundwater (Fig. 29). The percent of area covered by high intensity urban land cover was the third most influential variable. In general, riverscape alterations measured at the network scale, rather than the local scale, were most influential in predicting the occurrence of Sheepshead minnow. Predictions from the model revealed high probability of occurrence, and consequently high habitat suitability, for Sheepshead minnow in the mainstem Brazos River in close proximity to Possum Kingdom Reservoir, Granbury Reservoir, and Whitney Reservoir. The Double Mountain Fork of the Brazos River just downstream of Lake Alan Henry also showed high habitat suitability (Fig. 29).

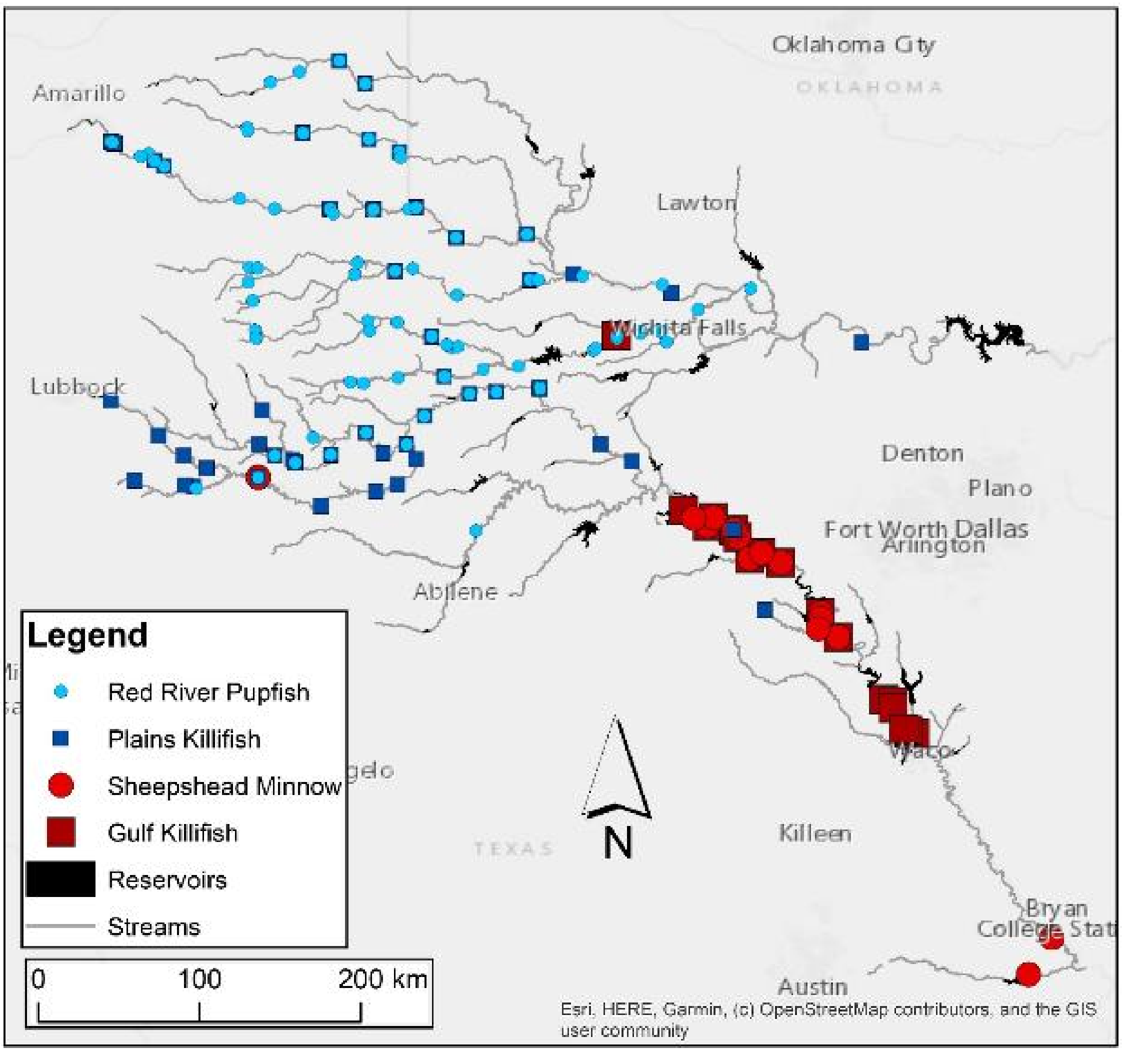

Fig. 28. Fish occurrence data used in the development of ecological niche models

The most influential variables in the Gulf killifish model were related to land cover and land use, groundwater input, and stream size. The most influential variable was the estimated phosphorous yield in the upstream drainage network, followed by the percent of base flow contributed by groundwater (Fig. 30). The percent of the upstream network coved by cultivated crops was the third most influential variable and was slightly more influential than the area of the upstream network drainage area. As with the Sheepshead minnow model, riverscape alterations measured at the network scale, rather than the local scale, were most influential in predicting the occurrence of Gulf killifish. Predictions from the model revealed high probability of occurrence, and consequently high habitat suitability, for Gulf killifish in the mainstem Brazos River in close proximity to Possum Kingdom Reservoir, Granbury Reservoir, and Whitney Reservoir. The Wichita River just downstream of Diversion Reservoir also showed high habitat suitability (Fig. 31).

These results collectively support the idea that reservoirs may act as invasion origin locations for stream fishes in general (Johnson et al. 2008) and Great Plains ecosystems in particular (Gido et al. 2004). Although to our knowledge no documented stocking history of either *C. variegatus* nor *F. grandis* are reported for the Brazos or Red River basins, coastal fishes including Red Drum were stocked into Kemp Reservoir in the Red River Basin and Tradinghouse Creek Reservoir in the Brazos River Basin (TPWD 2021). Both of these stocking locations are in close proximity to the areas of high probability of occurrence for both species studied here. Howells (2001) reviewed non-native fish introductions in Texas and discussed both *C. variegatus* and *F. grandis* introductions as the result of contaminations with targeted sportfish. Howells (2001) also noted that anglers in the Colorado River basin admitted to purposely introducing *F. grandis* as a forage species for local gamefish. It is possible that both accidental (e.g., Wellemeyer et al. 2016) and purposeful spread of these species in and around reservoirs is a major driver for their occurrence.

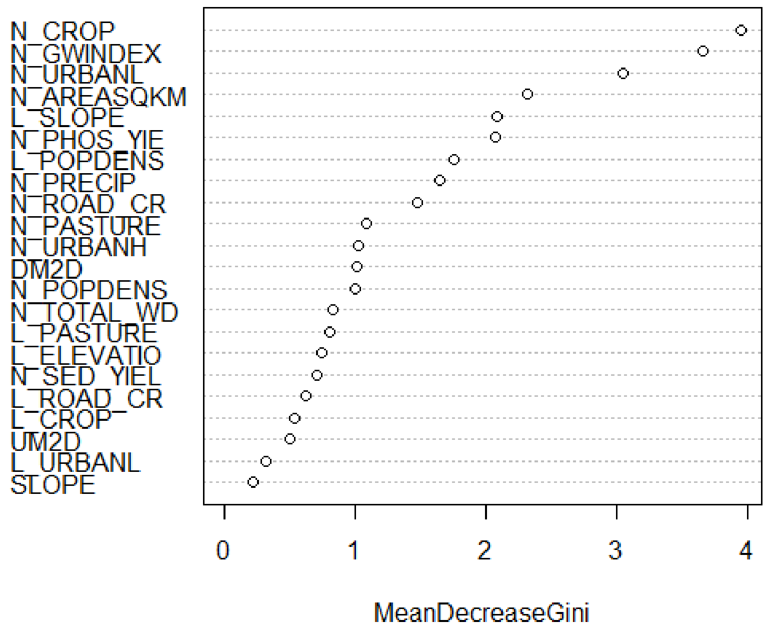

Fig. 29. Variable importance plot for the random forest model used to predict occurrence of Sheepshead Minnow (*C. variegatus*). See Table 1 for variable definitions and descriptions.

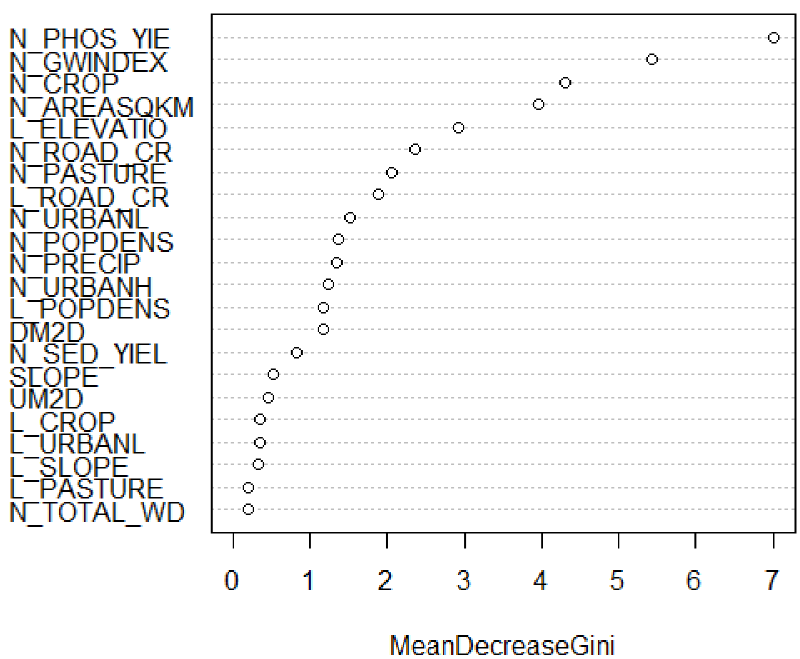

Fig. 30. Variable importance plot for the random forest model used to predict occurrence of Gulf killifish (*F. grandis*). See Table 1 for variable definitions and descriptions.

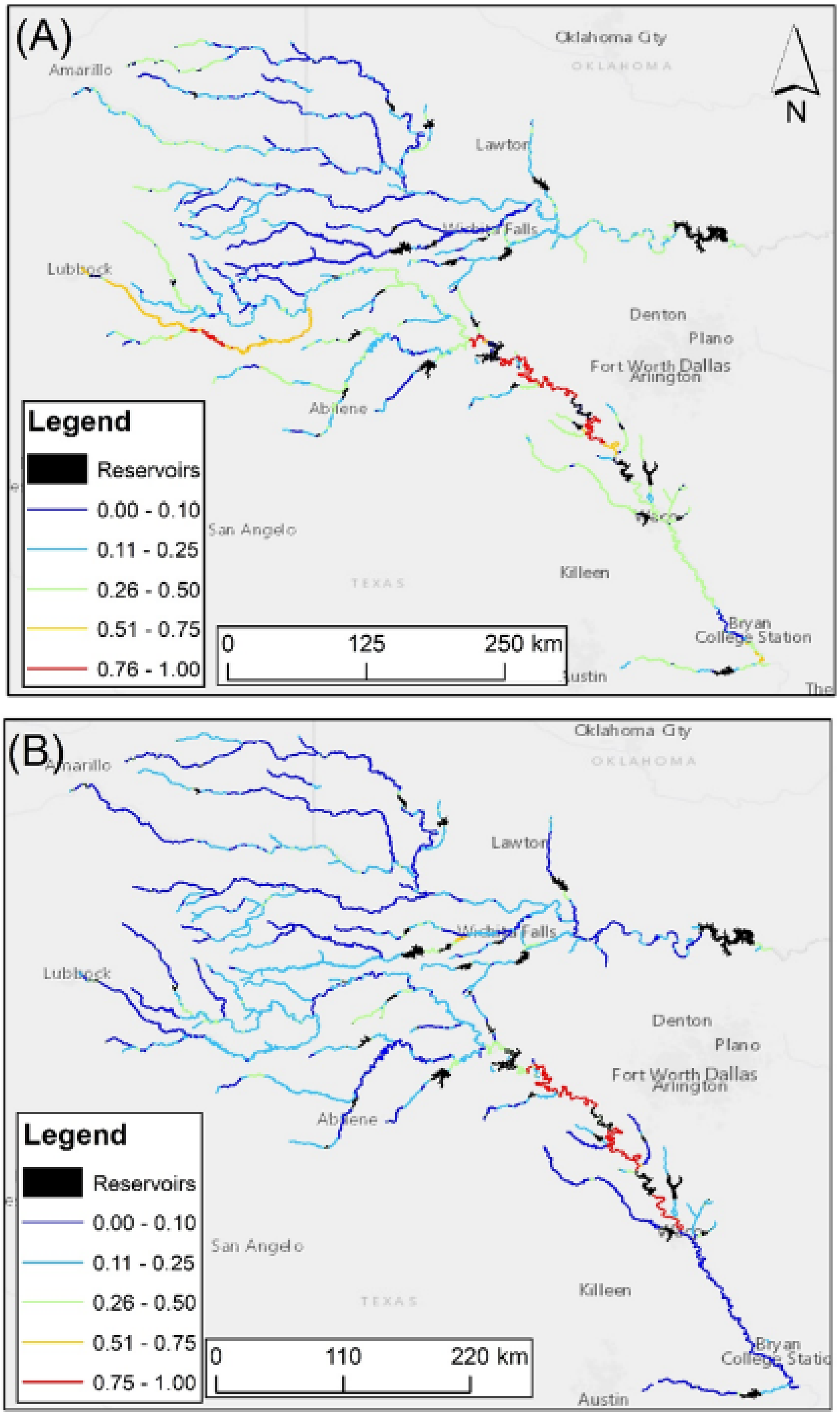

Fig. 31. Predicted probability of occurrence of for (A) *Cyprinodon variegatus* and (B) *Fundulus grandis* in the Brazos and Red river basins. Warm colors (orange and red) represent higher probability of occurrence.

CONCLUSIONS

Based on our surveys using telephone interviews and personal visits to live bait shops within the study area, we were unable to find evidence for the live bait industry being a main pathway for introduction of Sheepshead minnow and Gulf killifish into water bodies of the Middle and Upper reaches of Brazos River and tributaries of the Upper Red River basins. Our surveys reported native species such as Golden shiners and Fathead minnows as the most common species sold as live baits. The Goldfish and Black salty which are not native to the Brazos River, were sold as live bait in bait shops of the Middle and Upper Brazos River. Generally, the live bait shop owners and/or employees did not appear to have knowledge about our two targeted invasive cyprinodontids within the study area.

Although in low abundance, we did collect the two targeted cyprinodontid species in sites of the Middle Brazos River where they were collected in past surveys (Wilde 2012 unpublished data, Cohen et al. 2012). Sites included segments of the Brazos River between Lake Brazos above Waco and below Possum Kingdom Lake. These sites are near towns with urban development and pasture-land use, with high traffic of recreational fisheries because their closeness to the lakes. Based on projections of species distributions generated by the Random Forest models Task # 4), suitable habitats for invasions of these cyprinodontids are those near lakes/reservoirs, which could link introduction due to recreational fisheries in these places.

Our results are informative of the current status of the aquaculture industry in the Brazos and Red River basins. While we cannot inform how these two invasive species (Sheepshead minnow and Gulf killifish) were introduced into these aquatic systems, we confirmed their occurrence in sites of the Middle Brazos River which rises awareness for continuing monitoring these populations to evaluate potential upstream distribution expansion and ecological impacts in native cyprinodontids.

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