



# The ecological niche of native and invasive fish congeners in Texas streams: evidence from morphology, stable isotope analysis, and stomach contents analysis

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**Abstract** Freshwater invasions have occurred worldwide with significant ecological, evolutionary, and economic impacts on recipient ecosystems. Researchers now have access to a combination of approaches to better identify their potential ecological impacts on recipient communities. We used an integrative approach based on morphological traits, stomach contents, and stable isotope ratios ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) to examine multiple dimensions of the niche of native Texas cyprinodontids, Red River pupfish (*Cyprinodon rubrofluviatilis*) and plains killifish (*Fundulus zebrinus*), and their sympatric invasive congeners sheepshead minnow (*C. variegates*) and gulf killifish (*F. grandis*). We analyzed specimens from the Brazos River and Red River basins in Texas

collected during multiple surveys. Because of their phylogenetic relatedness, we predicted high overlap in the feeding habits and habitat-use between native and invasive congeners. A principal component analysis performed on 24 morphological traits associated with habitat-use and feeding ecology suggested some overlap on trait space occupied by the two *Cyprinodon* species, but no overlap between the two *Fundulus* species. High overlap on dietary and isotopic niche space was observed between the native and invasive *Cyprinodon* congeners. There was a large degree of seasonal variation in all four species stomach contents and isotopic signatures, which could be partially explained by low sampling sizes across certain seasons. While species differed in their trait space, similarities in the feeding ecology between cyprinodontid congeners could lead to negative ecological interactions related to food resources depending on the time

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of year. Our findings highlight the needs for continued monitoring of invasive cyprinodontids to prevent their spread into upstream reaches of the Brazos River to ensure conservation of native congeners.

**Keywords** Brazos River · *Cyprinodon* · Conservation · Freshwater invasion · *Fundulus* · Isotopic niche

## Introduction

Invasive species are currently one of the greatest threats to global biodiversity (Dudgeon et al. 2006) as they can have devastating ecological impacts spanning all levels of biological diversity. However, predicting their impacts on recipient ecosystems can be difficult (Crystal-Ornelas and Lockwood 2020). Examining different dimensions of an invasive species niche is one approach to better understand their invasion potential and predict ecological impacts on recipient communities (Bodey et al. 2011; Azzurro et al. 2014; Pacioglu et al. 2019; Calizza et al. 2021). Furthermore, quantifying their niche dimensions (e.g., diet, habitat-use, life history attributes; Pianka et al. 2017) relative to native species, may help elucidate the mechanisms underlying their establishment as well as their potential impacts. Successful invasions can occur by exploiting a novel niche within a community (i.e., empty niche hypothesis) or overlapping with one or more species in that recipient community (i.e., niche replacement hypothesis, Ricciardi et al. 2013). Species exploiting a novel niche are hypothesized to be able to establish populations more easily and create novel interspecific interactions (e.g., predation) leading to the declines of native species (Carpenter et al. 1985; Keitt et al. 2002; Hays and Conant 2007; Azzurro et al. 2014; Walsh et al. 2016). Niche overlap between native and non-native species can also result in antagonistic interactions (e.g., competition) impacting resource utilization within the recipient community (Ricciardi et al. 2013; Azzurro et al. 2014). Negative ecological interactions caused by invasive species can lead to displacement of native species with serious consequences on fitness, declines in native populations, or even extirpation (Gause 1934; Pfennig and Pfennig 2009).

Trait-based approaches have been used to make inferences about the potential for antagonistic

interactions between native and invasive species, as morphological similarity is assumed to capture niche similarity (Ricklefs and Miles 1994; Azzurro et al. 2014; Elleouet et al. 2014). Morphological traits have been used as proxy to reflect an organism's ecology including both their habitat-use and feeding ecology (Ricklefs and Miles 1994; Schalk et al. 2018; Luger et al. 2020). Using external morphology to infer niche space in invasive Mediterranean Sea fishes, Azzurro et al. (2014) found that successful invaders diverged in their morphological traits from native species and occupied novel niches in the recipient community. Although trait values can be plastic relative to other factors that can promote invasion success (Elleouet et al. 2014), trait-based approaches can complement other methods that quantify niche dimensions such as diet (i.e., stomach contents) and stable isotope analysis (e.g., carbon and nitrogen ratios) (de Carvalho et al. 2019; Minder et al. 2020; Bissantini et al. 2020). Stomach contents and stable isotope analyses can provide complementary aspects of an invasive species' dietary niche by allowing taxonomic resolution of food resources being exploited between native and invasive species as well as long-term perspectives on their trophic ecology (Pacioglu et al. 2019; de Carvalho et al. 2019; Marufu et al. 2018). Guzzo et al. (2013) used both stomach contents and stable isotopes to investigate ecological niche overlap between two perciforms (white perch *Morone americana* and yellow perch *Perca flavescens*) in Lake Erie. Their results suggested that overlap in dietary resources between invasive white perch contributed to the decline of the native yellow perch due to the increased competition for food resources.

Within Texas, two species of coastal cyprinodontid species, sheepshead minnow (*Cyprinodon variegatus*) and gulf killifish (*Fundulus grandis*), have been introduced outside of their native range with significant impacts on their native congeners in introduced basins (Echelle and Connor 1989; Cheek and Taylor 2015). *Cyprinodon variegatus* has been introduced into the Pecos River and Leon Springs where it hybridized with native congeners, Pecos pupfish (*C. pecosensis*) and Leon Springs pupfish (*C. bovinus*) (Echelle and Connor 1989; Echelle and Echelle 1994). The resulting hybrid offspring replaced genetically pure native *Cyprinodon* throughout most of their range (Hubbs et al. 2008; Wilde and Echelle 1992). These hybridization events resulted in *C. pecosensis* being listed as

state threatened and *C. bovinus* being listed as federally endangered (Wilde and Echelle 1992). *Fundulus grandis* was introduced into the Pecos and Rio Grande rivers with significant impacts to its native congener, plains killifish (*F. zebrinus*) (Hillis et al. 1980; Rhodes and Hubbs 1992; Cheek and Taylor 2015). Studies suggest that populations of native *F. zebrinus* have declined due to competition for food resources as well as direct predation from *F. grandis* (Cheek and Taylor 2015; Vaughan et al. 2016). While both species have been shown to negatively impact native populations of cyprinodontids within Texas, data quantifying the degree of niche overlap between these invasive cyprinodontids in relation to their native congeners are lacking. With the recent introduction of *C. variegatus* in the Brazos River (Wilde 2015) and *F. grandis* in the Wichita River (Robertson et al. 2019), it has become important to quantify their niche dimensions in inland Texas basins relative to their native congeners, which can be useful for predicting future invasion impacts.

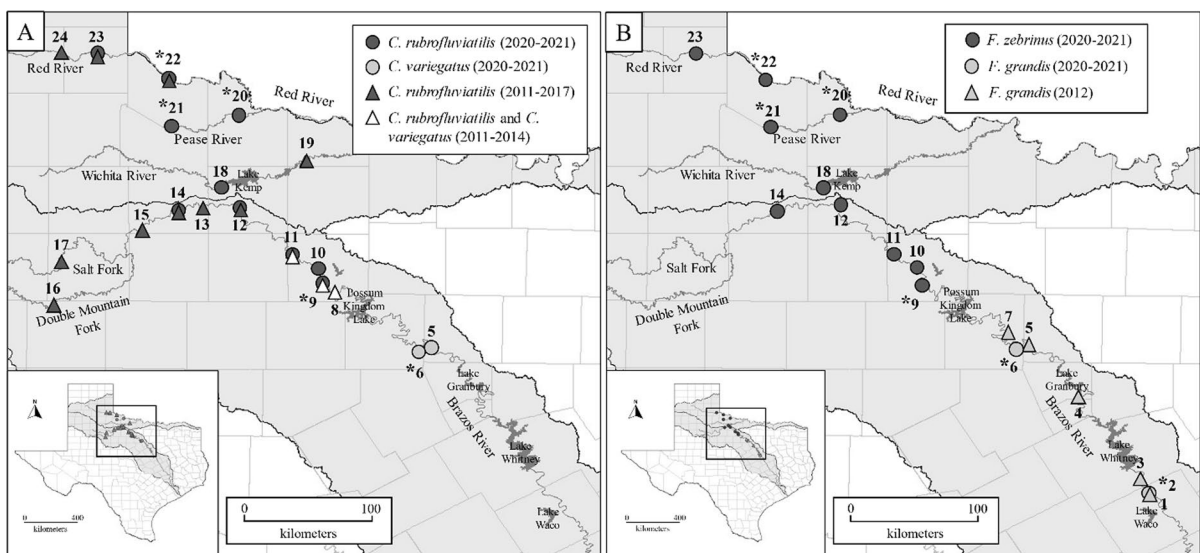
Here we used an integrative approach based on morphology, stable isotope, and stomach contents analyses to investigate ecological niches of the invasive *C. variegatus* and *F. grandis* and their native

congeners Red River pupfish (*C. rubrofluvialtilis*) and plains killifish (*F. zebrinus*) in the Brazos and Red River basins, Texas. Given their morphological similarities, phylogenetic relatedness, and past history of impacts within introduced basins, we hypothesized that the invasive species would overlap in their habitat-use and feeding ecology with their native congeners (i.e., the niche replacement hypothesis). Utilizing a more holistic approach to investigate several aspects of an invasive species niche may be necessary to better understand and predict invasive species potential future impacts.

## Materials and methods

### Study system and fish surveys

We collected focal fish species from sites within the Brazos River (between 31° 41' 32.2" N, 97° 14' 58.4" W and 33° 32' 53.4" N, 99° 39' 37.8" W) and upper Red River basins (between 33° 52' 09.4" N, 98° 50' 21.6" and 34° 34' 1.3" N, 100° 11' 45.6") in Texas (Fig. 1). Sites in the Brazos River extending from Lake Brazos in Waco upstream to Possum Kingdom



**Fig. 1** Locations of focal cyprinodontid species in the Brazos River and Red River basins (grey highlighted). **A** Survey locations for *Cyprinodon* species based on collections made in 2020–2021 (circles) and by Wilde 2011–2017 (triangles). **B** Survey locations for *Fundulus* species based on collections

made in 2020–2021 (circles) and by Labay et al. (2013) in 2012 (triangles). White triangles represent locations that contained both native (*C. rubrofluvialtilis*) and invasive (*C. variegatus*) species. Asterisk (\*) indicates locations where basal resources were collected

Lake are considered part of the middle Brazos River; while sites upstream of Possum Kingdom Lake are considered part of the upper Brazos River (Mayes et al. 2019). Twenty-two different sites (middle Brazos River  $n=6$ , upper Brazos River  $n=10$ , Red River  $n=6$ ) were sampled seasonally in summer 2020 (June and July), fall 2020 (September and October), winter 2020/2021 (December and February) and spring 2021 (March and May). Fishes were sampled using a seine net (6 m long  $\times$  1.5 m deep, 5 mm mesh size). A minimum sampling effort of 10 seine hauls were conducted at both upstream and downstream locations from the access point at each site. However, we conducted as many seine hauls as needed to cover all available habitats within the transect (transect length =  $\sim$ 500 m; seine hauls ranged from 10 to 20, total yielded: 401 seine hauls). All individuals of focal invasive species and, when available, a maximum of 30 individuals of each native species were retained for laboratory analysis.

Once collected, specimens were euthanized using clove oil. Following euthanasia, a portion of the fish ( $\sim$ 10 individuals) were preserved in ice, another portion in 90% ethanol, and another in 10% formalin solution. All preserved individuals were then transported to the Aquatic Ecology Lab at Stephen F. Austin State University for analysis of morphology, stable isotopes, and stomach contents. Individuals stored in ice and 90% ethanol were used for stable isotope analysis because formalin preservation has been shown to alter carbon and nitrogen signatures (Arrington and Winemiller 2002). The goal for preserving samples in ice and 90% ethanol was to examine a priori whether the preservation methods would yield different values of isotopic signatures given that all our samples for the historical surveys were preserved in ethanol. Our 2020–2021 surveys yielded a relatively low number of invasive species. Therefore, to supplement this low number of invasive specimens, we used specimens of *Cyprinodon* species collected by Wilde (2015) and *F. grandis* collected by Labay et al. (2013) in the Brazos River within our study area. Wilde (2015) samples were collected in different months between 2011 and 2014 at locations in the upper Brazos River, we also obtained additional samples collected by Wilde from 2015 to 2017 (unpublished data) in similar locations. Labay et al. (2013) collections were made in 2012 at locations in the middle Brazos River (Fig. 1). All specimens collected by Wilde (2011–2017) and

Labay et al. (2013) were preserved in 90% and 70% ethanol, respectively.

### Morphological analysis

In total, 431 fish specimens (*C. rubrofluviatilis*  $n=212$ , *C. variegatus*  $n=35$ , *F. zebrinus*  $n=168$ , *F. grandis*  $n=16$ ) collected between summer 2020 and spring 2021 were used for morphological analysis. In addition, 310 individuals from past surveys (*C. rubrofluviatilis*  $n=194$ , *C. variegatus*  $n=72$ , *F. grandis*  $n=44$ ) were also used for morphological analysis. Measurements were made to the nearest 0.01 mm using a digital caliper. We measured 24 morphological traits (see Suppl material Table S1) associated with food acquisition, locomotion, and habitat-use (Montaña and Winemiller 2013; Montaña et al. 2020). Traits associated with food acquisition included: gape width and snout mouth protrusion. Traits related with habitat-use and locomotion included: standard length, head length, head height, snout length, body depth and width, peduncle depth, peduncle width, peduncle length, dorsal length and height, anal length and height, caudal depth and length, pectoral length and height, and pelvic length and height. Traits associated with both food acquisition and locomotion included: interorbital distance, eye position, and eye diameter (Bower and Winemiller 2019). All distances measured were taken as the straight-line distance between points (Winemiller 1990). To remove influence of body size, linear morphological distances were transformed into ratios following the protocol outlined by Montaña and Winemiller (2013). While ratios of body size can introduce allometric bias into shape analysis, when using single class of specimens, allometric influence should be negligible to interpret interspecific morphological comparisons (Winemiller 1990; Montaña and Winemiller 2013).

### Stable isotope analysis

Carbon ( $\delta^{13}\text{C}$ ) values are assumed to reflect the basal resources assimilated into consumer tissue, while nitrogen ( $\delta^{15}\text{N}$ ) values reflect vertical trophic position of consumer (Montaña et al. 2020). In total, 347 fish specimens (*C. rubrofluviatilis*  $n=146$ , *C. variegatus*  $n=33$ , *F. zebrinus*  $n=152$ , *F. grandis*  $n=16$ ) collected between summer 2020 and spring 2021 were used for analysis of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . Our

surveys yielded a very low number of *F. grandis* (summer  $n=1$ , fall  $n=0$ , winter  $n=6$ , spring  $n=9$ ) and *C. variegatus* (summer  $n=0$ , fall  $n=6$ , winter  $n=15$ , spring  $n=12$ ). To complement our samples size for stable isotope analysis, we analyzed 170 tissue samples from individuals collected from past surveys (*C. rubrofluviatilis*  $n=47$ , *C. variegatus*  $n=79$ , *F. grandis*  $n=44$ ). Tissues for stable isotope analysis were prepared following the protocols by Arrington and Winemiller (2002). For small size specimens, the entire specimen (minus the gut) was processed for analysis. Prior to analysis, all individuals were soaked in deionized water for 60 min to remove any excess salt, mineral, or debris. Once rinsed, gut contents were removed and placed into a vial containing ethanol for later analysis. Individuals were dried in a drying oven at 60 °C for 48 h, ground into a fine powder using a sterilized mortar and pestle and stored in a sterilized glass vial. For each fish sample, a subsample 2.0–2.5 mg of powder was weighed and packaged into an Ultra-Pure tin capsule (Costech, Analytical Technologies Inc.). Samples were sent to the Analytical Chemistry Laboratory, Institute of Ecology, at the University of Georgia for analysis of stable isotope ratios of carbon ( $^{13}\text{C}/^{12}\text{C}$ ) and nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) isotope signatures using a Delta V mass spectrometer. Isotope ratios are reported in parts per thousand (‰) relative to standards in delta ( $\delta$ ) notation, as expressed according to the equation:

$$\delta X = \left[ \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \right] \times 1000$$

where X is  $^{13}\text{C}$  or  $^{15}\text{N}$ , and R is the ratios  $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$  (the ratio of heavy and light stable isotope of carbon and nitrogen respectively). The standard material for carbon ( $\text{d}^{13}\text{C}$ ) was Vienna PeeDee Belemnite (VPDB) limestone and atmospheric nitrogen ( $\text{N}_2$ ) was the standard for nitrogen ( $\text{d}^{15}\text{N}$ ). Precision of the isotopic analysis was  $\leq 0.11\text{‰}$  measured as the standard deviation among animal reference samples. Basal resources including leaf litter ( $n=18$ ), algae ( $n=17$ ), macrophytes ( $n=6$ ), periphyton ( $n=5$ ), grass ( $n=3$ ), and sediment ( $n=17$ ) were collected when available and used for stable isotope analysis to investigate production carbon sources being utilized by focal species. Basal resource samples were collected at select locations in the Brazos River and Red River (Fig. 1) during our sampling in the fall 2020, winter 2020,

and spring 2021 seasons. Resources were not collected in summer 2020 season because of the low collection of focal invasive species (*C. variegatus*  $n=0$ , *F. grandis*  $n=1$ ). Samples of basal resources were not available from historical surveys. Basal resource samples were prepared for stable isotope analysis following the same protocol used for fish tissues (Arrington and Winemiller 2002).

### Dietary analysis

Dietary analysis via examination of stomach contents was used to determine the abundance and type of prey consumed by focal species. In total, 725 fish specimens were examined and included samples from summer 2020 to spring 2021 (*C. rubrofluviatilis*  $n=265$ , *C. variegatus*  $n=59$ , *F. zebrinus*  $n=216$ , *F. grandis*  $n=16$ ) and from past surveys (*C. rubrofluviatilis*  $n=53$ , *C. variegatus*  $n=72$ , *F. grandis*  $n=44$ ). Fish specimens were dissected, stomachs removed and examined under a dissection microscope (Armscope Stereo Microscope). Prey items were identified to the lowest taxonomic level possible using taxonomic keys (McCafferty 1981; Merritt et al. 2008). Food items were then placed into broader taxonomic categories for use in calculations of dietary niche breadth and niche overlap. Such categories included: detritus, algae, plant material, sand/gravel, microplastics, Diptera, Trichoptera, Odonata, Hymenoptera, Coleoptera, Hemiptera, Megaloptera, Lepidoptera, Plecoptera, Ephemeroptera, unidentified insect parts, Nematoda, Annelida, Ostracoda, Gastropoda, fish parts, and microorganisms. Both volume and abundance of prey items consumed were recorded (Montaña and Winemiller 2013).

### Statistical analyses

Morphological and dietary data were used to investigate the niche dimensions of each species within selected river basins. Both morphological trait space and isotopic niche space were estimated using standard ellipse areas based (SEA) on Bayesian inference ( $\text{SEA}_B$ ) (Jackson et al. 2011; Montaña et al. 2020). The Bayesian approach for estimating SEA generates an area distribution that reflects the uncertainty associated with the sampling process that may otherwise bias SEA results (Jackson et al. 2011; Montaña et al. 2020). To calculate  $\text{SEA}_B$  for morphological



data, log-transformed morphological ratios were used in a principal components analysis (PCA) using the ‘vegan’ package in Rstudio version 4.0.5 (Oksanen et al. 2017). Results from PCA were used to make visual comparisons of species trait distribution within a multivariate space and to identify patterns among morphological variables (Montaña and Winemiller 2013). The scores from PC1 and PC2 were extracted and used to calculate  $SEA_B$ . Biplots of  $\delta^{13}C$  and  $\delta^{15}N$  values of basal resources and cyprinodontids were used to depict trophic structure and isotopic variation of native and invasive species. Given the low number of isotope samples collected in some seasons, we originally pooled the isotopic data across all four seasons to make stronger inferences about total resource overlap of these species (see Suppl material Fig. S2). A two-tailed t-test was used to test significant differences in mean  $\delta^{13}C$  and  $\delta^{15}N$  between congener pairs. However, there was seasonal variation in  $\delta^{13}C$  and  $\delta^{15}N$  values for each species. To address temporal variation in species diets, we created biplots of  $\delta^{13}C$  and  $\delta^{15}N$  values and calculated standard ellipse areas ( $SEA_B$ ) based on Bayesian Inference for both *Cyprinodon* and *Fundulus* species during each season. The  $SEA_B$  distribution was meant to reflect the morphological and isotopic niche space of each species.

Mean differences in multivariate trait space between native and invasive congeners were assessed for significance using permuted multivariate analysis of variance (PERMANOVA) based on Euclidean distance dissimilarity using the ‘vegan’ package in Rstudio version 4.0.5 (Schalk et al. 2018; Montaña et al. 2020). Overlap in  $SEA_B$  between congeneric species was calculated as the proportion of overlap area between both species divided by the total area occupied by both species (i.e.,  $(SEA_1 + SEA_2) - SEA_{12}$  overlap). Analysis of SEAs was conducted in RStudio version 4.0.5 using the ‘SIBER’ package (Jackson et al. 2011). The proportion of overlap in morphological and isotopic niche space between native and invasive congeners was used to make inferences about the potential for negative interspecific interactions. Where the larger the degree of overlap, the greater the potential for negative ecological interactions.

To further assess whether native and invasive species exhibit convergent patterns in dietary niches, we estimated the volumetric proportions of food items

used by each species (Montaña and Winemiller 2013). Volumetric proportions were estimated as described by Winemiller (1990). Diet diversity was estimated using Levins’ (1968) index of niche breadth (also called niche width or niche size), values closer to 1 represent a wide (generalist) niche breadth (Montaña and Winemiller 2009):

$$B' = \left( \sum_{i=1}^n P_i^2 \right)^{-1}$$

where  $B'$  is niche breadth,  $P_i$  is the volumetric proportion of food items  $i$  in the diet, and  $n$  is the total number of food items in the diet. Diet similarities was calculated using Pianka’s (1973) symmetrical measure of niche overlap. In this index, values range from 0 (no overlap) to 1 (complete overlap):

$$\Theta_{jk} = \left( \sum_{i=1}^n P_{ij} * P_{ik} \right) / \left( \sum_{i=1}^n P_{ij}^2 * P_{ik}^2 \right)^{1/2}$$

where  $\Theta_{jk}$  is symmetrical niche overlap between species  $j$  and  $k$ ,  $P_{ij}$  is the proportion of the resource  $i$  used by species  $j$ ,  $P_{ik}$  is the proportion of resource  $i$  used by species  $k$ , and  $n$  is the total number dietary item categories. Similar to our stable isotope analysis, indices were calculated for each season to better address temporal variation in species dietary habits.

## Results

### Cyprinodontid distribution

In our 2020–2021 surveys, we did not find overlap in the distribution of invasive *C. variegatus* and *F. grandis* and their native congeners. We did not collect either invasive species upstream of Possum Kingdom reservoir (Fig. 1). In our contemporary study, *C. variegatus* was only collected at two sites both of which were in the middle Brazos River, downstream of Possum Kingdom reservoir. Similarly, *F. grandis* was only collected at two sites located exclusively in the middle Brazos River. *Cyprinodon variegatus* and *F. grandis* ranges did appear to overlap as they were both collected at the same location in Palo Pinto County. Conversely, the native focal species, *C. rubrofluviatilis* and *F. zebrius* were

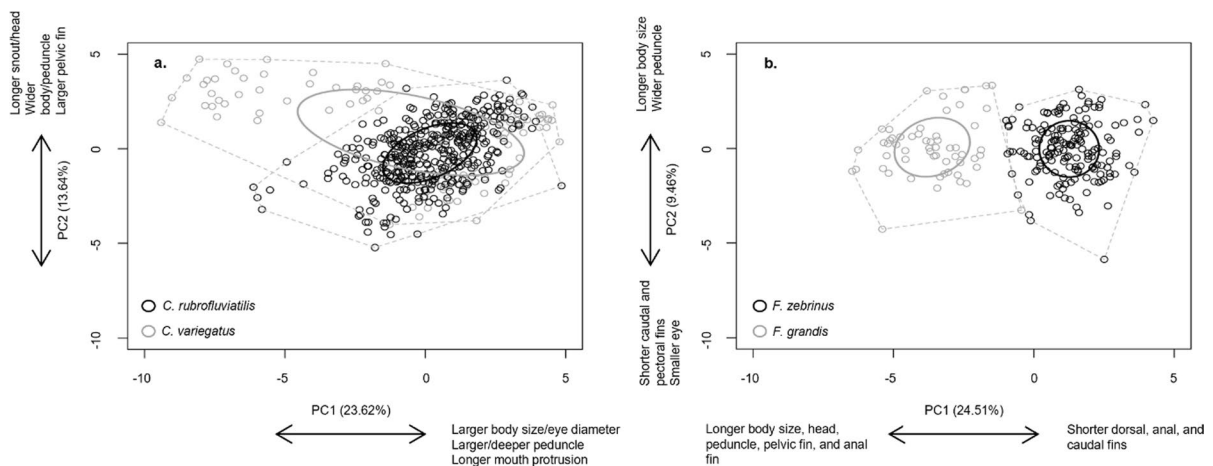
exclusively collected at locations in the upper Brazos River, upstream of Possum Kingdom reservoir, and throughout the Red River basin (Fig. 1). Samples used in this study that were collected by Wilde did contain *Cyprinodon* congeners collected concurrently at sites in the upper Brazos River. For this study, we were unable to obtain any samples of *Fundulus* species occurring concurrently, although previous studies have reported overlap in their range of distribution in the Red River basin.

### Cyprinodontid niche-trait space

The PCA of morphological traits revealed similarities between the two *Cyprinodon* species (Fig. 2a). The native, *C. rubrofluvialtilis* occupied a narrow trait space that was nested inside of the invasive *C. variegatus* trait space (Fig. 2a). The first two PCA axes explained 37.33% of the morphological variation (PC1=23.62%, PC2=13.64%; see Suppl material Table S3) and revealed divergence of fish based on traits associated with location and habitat-use (PC1), as well as a gradient of trophic ecology and habitat-use (PC2). The PC1 resulted in a gradient mainly associated with locomotion, with individuals with positive loadings having larger body size and eye diameter, larger and deeper peduncles, larger dorsal and anal fins, and a longer snout-mouth protrusion (Fig. 2a). The PC2 showed a gradient

reflecting traits associated with species trophic ecology and habitat-use. For example, individuals with positive loadings (e.g., *C. variegatus*) had a longer snout and head, smaller interorbital distance, wider peduncle and body, and larger pelvic fins (Fig. 2a). *Cyprinodon variegatus* occupied a larger area in the trait space (median  $SEA_B = 150.27$ , 95% CI [127.04, 185.34]) compared to *C. rubrofluvialtilis* (median  $SEA_B = 44.39$ , 95% CI [40.25, 49.48]) (Fig. 2a). *Cyprinodon* species occupied statistically different morphological trait spaces (PERMANOVA:  $F_{(1,508)} = 16.94$ ,  $p < 0.001$ ). Results of the percentage of  $SEA$  overlap suggest that while both species occupied statistically different morphological trait spaces, there was still some overlap in that trait space ( $SEA_{overlap}$  median = 28.30%, CI 95% [22.16%, 32.42%]).

The PCA of morphological traits of *Fundulus* species revealed no overlap in trait space occupied by *F. zebrinus* and *F. grandis* (Fig. 2b). The PC1 (24.51% of variation, see Suppl material Table S4) reflected differences between *F. zebrinus* and *F. grandis* on traits associated with body size and shape (Fig. 2b). Individuals of *F. zebrinus* had shorter dorsal, anal, and caudal fins, while *F. grandis* were on average larger in body size, head size, peduncle size, pelvic fins, and anal fins. The PC2 (9.46% of variation, see Table S4) contrasted traits associated with locomotion (Fig. 2b). Individuals with positive loading scores had



**Fig. 2** Principal component analysis of cyprinodontid species in the Brazos and Red Rivers based on 24 morphological traits for **a** *Cyprinodon* species and **b** *Fundulus* species. Polygons with dashed gray lines represent each species convex hull, each

point represents an individual sample. The standard ellipses area based on Bayesian inference ( $SEA_B$ ) are shown in solid lines (black = native species, gray = invasive species). Text below axes show morphological traits with highest loadings

a larger body size and wider peduncle, while individuals with negative loading scores had shorter caudal and pectoral fins, and smaller eyes. *Fundulus grandis* occupied a slightly larger trait space than *F. zebrinus* (*F. grandis*  $SEA_B$  median=39.45, 95% CI [31.17, 50.17], *F. zebrinus*  $SEA_B$  median=29.92, 95% CI [29.95, 35.51]) (Fig. 2a). *Fundulus* species occupied statistically different morphological trait space (PERMANOVA:  $F_{(1,204)}=56.77$ ,  $p<0.001$ ). Likewise, they showed almost no overlap in their standard ellipses' areas ( $SEA_{overlap}$  median=4.36%, 95% CI [1.12%, 8.88%]).

### Basal resources and species isotopic niches

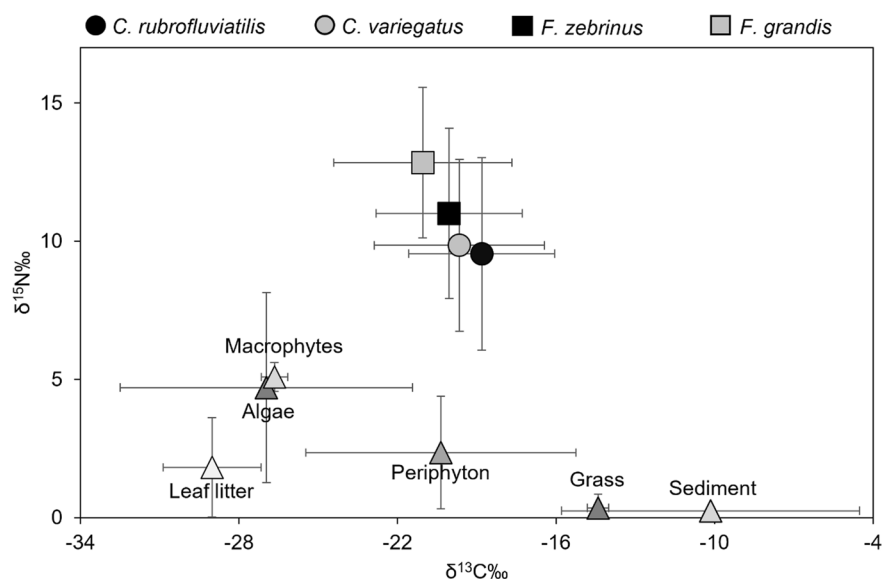
Values of  $\delta^{13}C$  ratios varied among basal resources, with ratios between  $-20.0$  and  $-29.0\text{‰}$ . With the exception of aquatic grasses ( $-14.4\text{‰}\pm 0.4$ ) which had enriched  $\delta^{13}C$ , all other basal resources collected had  $\delta^{13}C$  slightly enriched (leaf litter:  $-29.0\text{‰}\pm 1.9$ , algae:  $-27.0\text{‰}\pm 5.5$ , aquatic macrophytes:  $-26.7\text{‰}\pm 0.5$ , benthic algae:  $-20.4\text{‰}\pm 5.1$ ). Sediment samples were also  $\delta^{13}C$  enriched ( $-10.2\text{‰}\pm 5.6$ ) (Fig. 3).

While fish samples used for stable isotope analysis were preserved in both ethanol and ice, preservation type was not shown to significantly alter  $\delta^{13}C$  (t-test:  $t_{(454)}=-1.18$ ,  $p=0.24$ ) or  $\delta^{15}N$  values (t-test:  $t_{(454)}=0.42$ ,  $p=0.68$ ). There was seasonal variation in the values of  $\delta^{13}C$  and  $\delta^{15}N$  ratios. However,

*Cyprinodon* species showed some degree of overlap in their isotopic niches during each season (Fig. 4). *Cyprinodon rubrofluviatilis* had  $\delta^{13}C$  values between  $-28.2$  and  $-12.3\text{‰}$  and *C. variegatus* had  $\delta^{13}C$  values between  $-27.1$  and  $-15.8\text{‰}$  across all four seasons (see Suppl material Fig. S2). A two-tailed t-test revealed that *Cyprinodon* species did differ in their total  $\delta^{13}C$  values ( $t_{(204)}=2.32$ ,  $p<0.05$ ). Even with this variation, both species seem to be utilizing carbon pathways derived from algae and periphyton sources (Fig. 4). *Cyprinodon rubrofluviatilis* had highly variable isotopic niche size, with median  $SEA_B$  ranging from  $23.8\text{‰}^2$ , CI 95% [ $23.4\text{‰}^2$ ,  $54.6\text{‰}^2$ ] in the summer to  $141.8\text{‰}^2$ , CI 95% [ $111.2\text{‰}^2$ ,  $178.8\text{‰}^2$ ] in the fall (Table 1). Similarly, *C. variegatus* isotopic niche also varied, with median  $SEA_B$  ranging from  $8.5\text{‰}^2$ , CI 95% [ $5.8\text{‰}^2$ ,  $15.1\text{‰}^2$ ] in the summer to  $127.4\text{‰}^2$ , CI 95% [ $94.5\text{‰}^2$ ,  $172.2\text{‰}^2$ ] in the winter (Table 1). Overlap in isotopic niche space between *Cyprinodon* congeners was reflected by median  $SEA_{overlap}$  and ranged from 20.53%, CI 95% [16.06%, 28.76%] in the fall to 44.85%, CI 95% [32.50%, 53.90%] in the spring (Table 1).

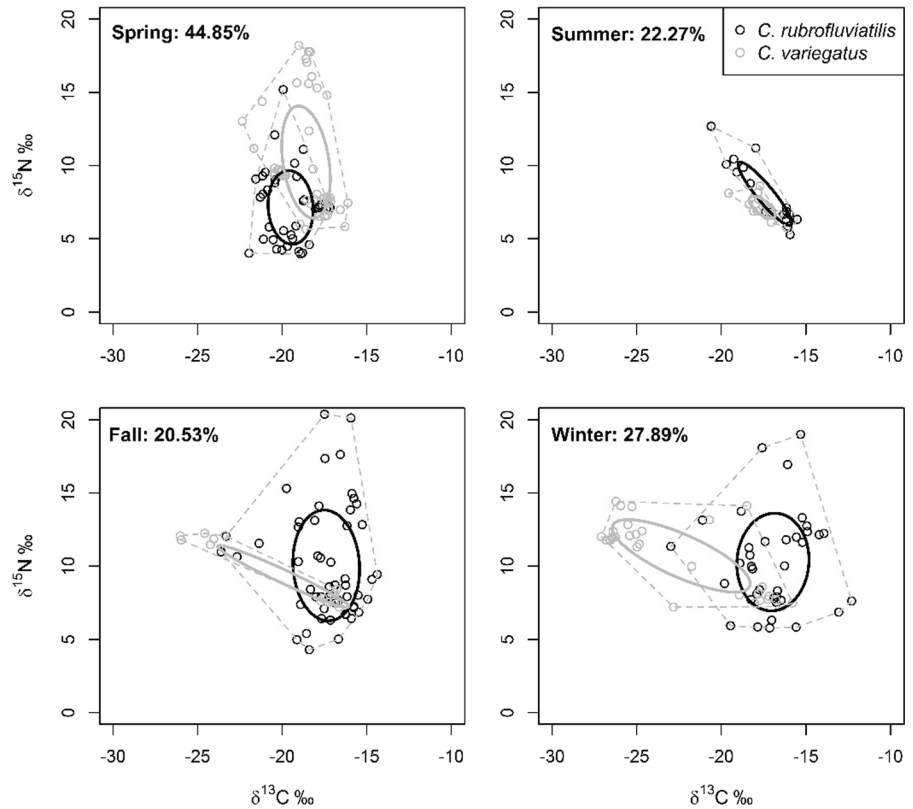
*Fundulus* species also had variation in their isotopic signatures seasonally (Fig. 5). In general, *Fundulus* species did not differ significantly in their  $\delta^{13}C$  values ( $t_{(107)}=-1.97$ ,  $p=0.06$ ) ranging between  $-27.6$  and  $-13.2\text{‰}$  in *F. zebrinus* and between  $-26.6$  and  $-14.6\text{‰}$  in *F. grandis* (see Suppl material Fig. S2), suggesting that they also utilized carbon

**Fig. 3** Biplot of  $\delta^{13}C$  and  $\delta^{15}N$  values for focal species (circles = *Cyprinodon* species, squares = *Fundulus* species, grey = invasive species, black = native species) and basal resources (triangles) collected at sites along the Brazos and Red Rivers. Values represent the mean  $\pm$  standard deviation





**Fig. 4** Biplot of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for *Cyprinodon rubrofluviatilis* (black) and *C. variegatus* (gray) by season (spring: March–May, summer: June–August, fall: September–November, and winter: December–February). Polygons with dashed lines represent each species convex hull. Standard ellipses area ( $\text{SEA}_B$ ) were based on Bayesian inference for each species are shown in solid lines, percentage reflect median percent overlap in  $\text{SEA}_B$  between *Cyprinodon* species during each season

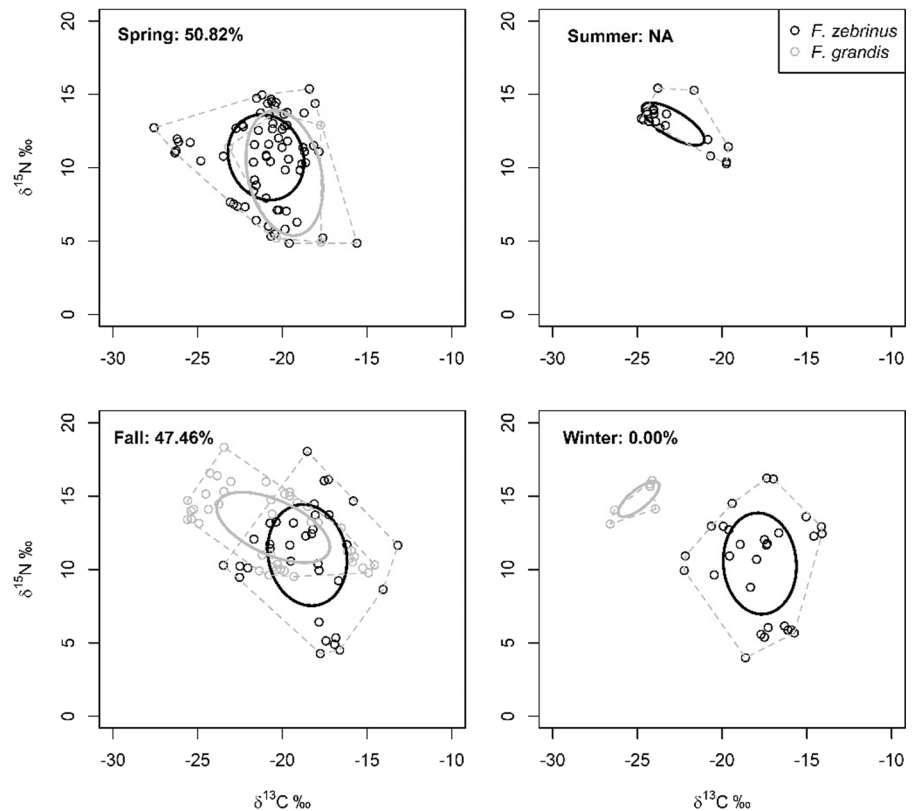


**Table 1** Standard ellipses area ( $\text{SEA}_B$ ) based on Bayesian inference with 95% confidence intervals for native and invasive cyprinodontid species shown by season. Median area of over-

lap ( $\text{SEA}_{\text{Overlap}}$ ) with 95% confidence intervals between congener pairs also shown by season

Season	Species	Number of Individuals (n)	$\text{SEA}_B$ median ( $\%e^2$ )	$\text{SEA}_B$ 95% CI $\pm$	$\text{SEA}_{\text{Overlap}}$ median (%)	$\text{SEA}_{\text{Overlap}}$ 95% CI $\pm$
Spring	<i>C. rubrofluviatilis</i>	38	64.4	49.7, 85.2	44.85	32.50, 53.90
	<i>C. variegatus</i>	46	98.9	80.3, 133.4		
	<i>F. zebrinus</i>	69	122.3	91.2, 151.7		
	<i>F. grandis</i>	5	174.3	93.6, 484.8		
Summer	<i>C. rubrofluviatilis</i>	17	23.8	23.4, 54.6	22.27	12.79, 39.48
	<i>C. variegatus</i>	16	8.5	5.8, 15.1		
	<i>F. zebrinus</i>	19				
	<i>F. grandis</i>	1				
Fall	<i>C. rubrofluviatilis</i>	54	141.8	111.2, 178.8	20.53	16.06, 28.76
	<i>C. variegatus</i>	16	64.0	42.4, 102.1		
	<i>F. zebrinus</i>	34	155.3	109.0, 219.9		
	<i>F. grandis</i>	43	126.4	97.4, 172.5		
Winter	<i>C. rubrofluviatilis</i>	37	136.6	98.1, 200.4	27.89	22.72, 34.17
	<i>C. variegatus</i>	30	127.4	94.5, 172.2		
	<i>F. zebrinus</i>	29	139.9	98.3, 204.6		
	<i>F. grandis</i>	6	22.0	9.0, 60.3		

**Fig. 5** Biplot of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for *Fundulus zebrinus* (black) and *F. grandis* (gray) by season by season (spring: March–May, summer: June–August, fall: September–November, and winter: December–February). Polygons with dashed lines represent each species convex hull. Standard ellipses area ( $\text{SEA}_B$ ) was based on Bayesian inference for each species are shown in solid lines, percentage reflect median percent overlap in  $\text{SEA}_B$  between *Fundulus* species during each season



pathways derived from algae and periphyton sources (Fig. 3).  $\text{SEA}_B$  and  $\text{SEA}_{\text{Overlap}}$  were unable to be calculated for *Fundulus* species during the summer due to the low number of *F. grandis* collected ( $n=1$ ). Unlike *Cyprinodon* species, *F. zebrinus* isotopic niche size remained relatively consistent, with median  $\text{SEA}_B$  ranging from  $122.3\text{‰}^2$ , CI 95% [ $91.2\text{‰}^2$ ,  $151.7\text{‰}^2$ ] in the spring to  $155.3\text{‰}^2$ , CI 95% [ $109.0\text{‰}^2$ ,  $219.9\text{‰}^2$ ] in the fall (Table 1). *Fundulus grandis* had more seasonal variation in isotopic niche size, with median  $\text{SEA}_B$  ranging from  $22.0\text{‰}^2$ , CI 95% [ $9.0\text{‰}^2$ ,  $60.3\text{‰}^2$ ] in the winter to  $174.3\text{‰}^2$ , CI 95% [ $93.6\text{‰}^2$ ,  $484.8\text{‰}^2$ ] in the spring (Table 1). Overlap in isotopic niche space of *Fundulus* congeners was also reflected by median  $\text{SEA}_{\text{overlap}}$  and ranged from 0.00%, CI 95% [0.00%, 0.05%] in the winter to 50.82%, CI 95% [22.15%, 76.00%] in the spring (Table 1).

#### Dietary patterns

For all four species, we found some variation in food items identified in the stomach contents across all

four seasons (Table 2). In general, all four species of cyprinodontids ingested large portions of organic detritus, algae, and sand/gravel. Both native and invasive *Cyprinodon* species, consumed high amounts of macroinvertebrates in the order Diptera. Compared to its native congener, *C. variegatus* ingested a larger portion of microcrustaceans in the class Ostracoda. Both *Cyprinodon* species appear to have a more specialized diet, with niche breadth values ( $B'_A$ ) ranging seasonally from 0.025 to 0.078 for *C. rubrofluviatilis* to 0.019 to 0.071 for *C. variegatus* (Table 2). *Cyprinodon* congeners showed a high degree of overlap in their diets as well, where niche overlap ( $\Theta_{ik}$ ) ranged from 0.751 in the spring to 0.998 in the summer (Table 2). Results may have been impacted by relatively low sampling sizes for certain seasons (*C. rubrofluviatilis*: summer, *C. variegatus*: summer, fall) as large sample sizes are typically required for a robust stomach contents analysis (Han et al. 2020).

Both native and invasive species of *Fundulus* also ingested high proportions of macroinvertebrates in the orders Diptera and Hemiptera, and several taxa within the class Crustacea. *Fundulus grandis*

**Table 2** Results for calculations of Levins' (1968) index of niche breadth ( $B'_A$ ) and Pianka's (1973) measure of niche overlap ( $\Theta_{jk}$ ) shown by season

Season	Species	Number of individuals (n)	Levins' niche breadth ( $B'_A$ )	Pianka's niche overlap ( $\Theta_{ik}$ )
Spring	<i>C. rubrofluviatilis</i>	119	0.078	0.751
	<i>C. variegatus</i>	51	0.045	
	<i>F. zebrinus</i>	103	0.052	
	<i>F. grandis</i>	9*	0.086	
Summer	<i>C. rubrofluviatilis</i>	22*	0.025	0.998
	<i>C. variegatus</i>	10*	0.019	
	<i>F. zebrinus</i>	19*	0.069	
	<i>F. grandis</i>	1*	0.042	
Fall	<i>C. rubrofluviatilis</i>	71	0.036	0.997
	<i>C. variegatus</i>	20*	0.053	
	<i>F. zebrinus</i>	52	0.137	
	<i>F. grandis</i>	44	0.086	
Winter	<i>C. rubrofluviatilis</i>	107	0.038	0.950
	<i>C. variegatus</i>	50	0.071	
	<i>F. zebrinus</i>	41	0.046	
	<i>F. grandis</i>	6*	0.102	

Asterisk (\*) means indicates seasons where samples were too small to make meaningful inferences

consumed larger portions of Odonata, Ephemeroptera, Nematoda, and Gastropoda, and fish compared to *F. zebrinus*. *Fundulus* species also appear to have relatively narrow dietary niche breadth ( $B'_A$ ). For instance, dietary niche breadth of *F. zebrinus* ranged from 0.052 in spring to 0.137 in fall, while in *F. grandis* niche breadth ranged from 0.042 in summer to 0.102 in winter (Table 2). The niche overlap of *Fundulus* species appeared to vary seasonally, with high overlap ( $\Theta_{ik}=0.849$ ) in fall compared to winter ( $\Theta_{ik}=0.289$ ). However, similar as *Cyprinodon* species, low sampling sizes for *Fundulus* species during certain seasons (*F. zebrinus*: summer, *F. grandis*: spring, summer, winter) may have impacted results as large sample sizes are typically required for robust assessments of stomach contents (Han et al. 2020).

## Discussion

Predicting the potential impacts of invasive species introductions can be difficult due to the diverse outcomes from such invasions (Ricciardi et al. 2013; Elleouet et al. 2014). Based on their morphological traits, we found little evidence to suggest that native and invasive cyprinodontids might overlap in their habitat-use. However, we did find support for high niche overlap for *C. variegatus* and *F. grandis* with

their native congeners along their dietary and isotopic niche dimensions during certain times of the year. Our contemporary field surveys indicate that the distribution of both *C. variegatus* and *F. grandis* appears to be restricted to the middle Brazos River, downstream of Possum Kingdom reservoir, where the presence of their native congeners is limited. We did not find native and invasive cyprinodontids congeners occurring in sympatry in middle Brazos River sites. However, this does not reduce the potential risks posed by *C. variegatus* and *F. grandis* to their native congeners within the Brazos and Red River basins due to their similarities in resource use across certain seasons and past history of invasion impacts within inland Texas basins (Echelle and Connor 1989; Cheek and Taylor 2015). Our findings indicate that if in sympatry, invasive cyprinodontids have the potential to impact native congener populations in the Brazos and Red River basins.

## Niche-trait divergence

Differences in body sizes between co-occurring competitors can help facilitate their coexistence within a system, as body size is a key trait that influences competitive ability and resource-use (Anaya-Rojas et al. 2021). Differences in overall body size was the main trait separating individuals of *Fundulus* species

in our study. *Fundulus grandis* is the largest species within its genus, reaching up to 18 cm in length (Ross 2001), while *F. zebrinus* typically reaches a maximum of 10 cm in length (Page and Burr 1991). The coexistence of these two *Fundulus* species may be facilitated by differences in body size, as they may partition resources (e.g., space, habitat) within the Brazos River basin. While these species are shown to utilize similar dietary resources, other aspects of their resource utilization within the Brazos River need to be investigated in the future to better understand intraspecific interactions that may facilitate coexistence.

*Cyprinodon* species exhibited low overlap in their niche trait space. Morphological differences between *C. rubrofluviatilis* and *C. variegatus* are primarily related to the scaling on their abdomen and coloration patterns (Hubbs et al. 2008; Wilde and Echelle 1997). While we were unable to determine any specific traits that differ between *C. rubrofluviatilis* and *C. variegatus*, there was a clear separation of *C. variegatus* into two distinct morphological groups. One group of larger individuals of *C. variegatus* almost completely overlapped in morphological space with *C. rubrofluviatilis*, while the other group of individuals did not. A similar pattern in body size was observed by Gunter (1950) in populations of *C. variegatus* from the Texas gulf coast, in which individuals within two distinct size classes were present throughout the year. They hypothesized that this pattern could be attributed to the long spawning season of this species, which allows for the presence of both large adults and small juveniles within a single population across the year (Gunter 1950). Differences in morphological traits between the native and invasive cyprinodontids could indicate potential differences in their habitat-use within the Brazos River which can favor coexistence. However, future studies assessing size structure and some aspects of *Cyprinodon*'s life history (e.g., reproduction) within this river basins could help to elucidate each invasive species' habitat-use throughout their lives and help confirm the morphological difference observed in this study.

#### Dietary and isotopic niche dimensions

General trends of our findings suggest that invasive cyprinodontids occupy similar isotopic and dietary niche space as their native congeners, with a

large degree of seasonal variation. Both *Cyprinodon* and *Fundulus* species appear to be utilizing carbon derived from benthic algae as their main source of energy. Within the Brazos River, algae are reported as the most common carbon source supporting consumers during low water flow (Zeug and Winemiller 2008). In our systems, benthic algae appear to be an important energy source for diet of all four cyprinodontids, and it may indicate that these species are feeding in shallow habitats with low flow. These four cyprinodontids are known for their affinity for shallow, isolated areas, and backwaters where there is little to no flow (Hillis et al. 1980; Johnson 1980; Minckley 1980). Such habitats may promote the production of benthic algae, increasing its contribution to consumer biomass over other carbon sources in these riverine environments. In their native coastal marine environments, *C. variegatus* and *F. grandis* have shown to assimilate carbon derived from C<sub>4</sub> marsh grasses (Romanek et al. 2000; Winemiller et al. 2007; Rezek et al. 2017), but plasticity in their dietary habits within inland ecosystems appear common. For many invasive species, such plasticity in novel environments is known to promote their persistence and spread throughout the system (Colautti et al. 2006).

*Fundulus* species appeared to differ in their  $\delta^{15}\text{N}$  signatures, especially during the winter, in which *F. grandis* had slightly higher values of  $\delta^{15}\text{N}$  compared to its native congener. *Fundulus grandis* appears to be more carnivorous than *F. zebrinus*, consuming a wider variety of fish and macroinvertebrates, a finding that agrees with Vaughan et al. (2016). *Fundulus grandis* had a large degree of seasonal variation in its isotopic signature and dietary niche breadth, such variation could imply that *F. grandis* has the ability switch dietary resources as they fluctuate seasonally. Dietary plasticity is known to be correlated with the persistent and success of invasive species such as *F. grandis* (Colautti et al. 2006). Other researchers also reported seeing variation in *F. grandis*' diet in introduced systems (Cheek and Taylor 2015; Vaughan et al. 2016). For example, Cheek and Taylor (2015) reported that *F. grandis* introduced into the Lower Pecos River, TX were directly competing for food with its native congener, *F. zebrinus*. Contrary to that, Vaughan et al. (2016) saw very little overlap in *F. grandis* and *F. zebrinus* stomach contents, stating that *F. grandis* was much more piscivorous than previously reported. While our study and results reported

by Cheek and Taylor (2015) may indicate fluctuating degrees of resource overlap between these congeners, Vaughan et al. (2016) suggest that *F. grandis* may also be impacting populations of *F. zebrinus* through direct predation as well. Even though niche overlap between *Fundulus* species seems to vary seasonally, *F. grandis* still poses a threat to *F. zebrinus* populations in the Brazos and Red rivers. Hoagstrom et al. (2010) discuss that invasive species are able to replace native species if they can grow quickly, invade already disturbed ecosystems, and are closely related to native species. *Fundulus grandis* within the Brazos and Red rivers fit all these criteria (Hoagstrom et al. 2010; Cheek and Taylor 2015; Mayes et al. 2019). That, coupled with *F. grandis* invasion history indicates that populations of *F. zebrinus* in the Brazos and Red River basins are still at risk for impacts should they come in contact with *F. grandis* in the future.

*Cyprinodon* species were confirmed to be omnivorous, consuming algae and macroinvertebrates, along with large amounts of sand and sediment (Harrington and Harrington 1961; Miller and Robison 2004). While there was some seasonal variation, native and invasive *Cyprinodon* species had extremely narrow dietary niche breadths and both appear to be dipteran larvae specialists (Harrington and Harrington 1961). Ecological specialization is often a mechanism that allows for resource partitioning and promotes the coexistence of species (Wilson and Yoshimura 1994). However, potential negative interactions could occur if two species are specializing in the same resource (Wilson and Yoshimura 1994). High dietary overlap between *Cyprinodon* species could result in reduction of fitness in native congener, lead to competitive displacement or potentially exclude the native *Cyprinodon* from the system if dietary resources become limiting. While stomach contents confirmed overlap of dietary resources, stable isotope analysis found high seasonal variation in isotopic overlap between *Cyprinodon* species. Further research is needed to confirm trends observed in *Cyprinodon* stomach contents while accounting for potential seasonal and spatial differences in *Cyprinodon* isotopic signatures.

#### Ecological implications

Currently, introduced populations of *C. variegatus* and *F. grandis* seem to be restricted to the middle

portion of the Brazos River, where they are unlikely to encounter their native congeners, but that does not mean that they do not pose a risk within this recipient basin. Previously, both invasive species have been collected in more northern locations, overlapping ranges with their native congeners in the Brazos and Red Rivers. The exact pathway of introduction of *C. variegatus* and *F. grandis* within Texas is still unknown. Until that pathway is identified and eliminated, invasive propagules still have the potential to reestablish populations upstream of Possum Kingdom reservoir. As shown from evidence in this study and in other inland basins in Texas, *C. variegatus* and *F. grandis* may have the potential to impact their native congeners within the Brazos and Red River basins if they were to come in contact again in the future.

Based on isotopic and dietary analyses, both native and invasive cyprinodontids revealed overlap in their trophic ecology, with a high degree of seasonal variation. Such overlap could influence resource-use and abundance of native populations of *C. rubrofluvialtilis* and *F. zebrinus*. Given the limited sample size for some seasons, we were unable to provide robust analysis of niche overlap across the entire year. However, we encourage future research in this line to help gain a more holistic understanding of potential interspecific interactions between these congener pairs and how that might vary between seasons. *Cyprinodon variegatus* also has been shown to hybridize with other *Cyprinodon* species in the past, therefore the fitness of the state listed threatened species *C. rubrofluvialtilis* (Birdsong et al. 2020) could be compromised as well. Future research should aim to identify mechanisms facilitating their spread throughout these focal basins in order to prevent future contact with *C. rubrofluvialtilis* and *F. zebrinus*. Managing established populations of invasive cyprinodontids within the middle Brazos River and preventing their spread into upstream locations is the best way to ensure the continued conservation of *C. rubrofluvialtilis* and *F. zebrinus* into the future. If species do come into contact again, a more in-depth look at the spatial and temporal variation in resource overlap should be done to confirm trends observed in this study.

Predicting the impacts of an introduced species within a community is very difficult. However, using a combination of methods as those applied in this study (morphology, stomach contents, and stable isotope analysis) can have broad implications for



assessing the potential impacts of invasive species. For instance, they can give valuable insight into an invasive species habitat-use and diet within an introduced system. Overlap in resource-use with invasive species can have drastic implications for members of the native community, mainly through the processes of competitive displacement and exclusion. Using a more holistic approach such as the one used here to investigate several aspects of an invasive species niche may be necessary to better understand and predict invasive species impacts in the future.

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**Author contributions** ES, CGM, KM, JP contributed to the study conception and design. ES and CGM contributed to material preparation and data collection. ES, CGM and CMS contributed to data analysis. The first drafts of the manuscript were written by ES with guidance of CGM and CMS. All authors contributed comments on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data availability** The data that support the findings of this study are included in the article; further inquiries can be directed to the corresponding author upon reasonable request.

## Declarations

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.

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