

Insights on the establishment of the invasive Sheepshead minnow (*Cyprinodon variegatus*) in the Brazos River

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ABSTARCT

Biological invasions have occurred worldwide with significant ecological, evolutionary, and economic impacts on recipient ecosystems. Researchers have been using a combination of approaches to better identify the invaders and the risk of the invasions in recipient communities. We used a novel integrative approach based on morphological traits, stomach contents, and stable isotope ratios ($\delta^{13}\text{C}$ & $\delta^{15}\text{N}$) to examine the size of the morphological space, as well as dietary and isotopic niche space of the native Texas State threatened Red River pupfish (*Cyprinodon rubrofluviatilis*) and invasive Sheepshead minnow (*C. variegatus*). We analyzed 74 individuals of *C. variegatus*, 248 individuals of *C. rubrofluviatilis*, and 50 potential hybrids that were collected from locations along the upper Brazos River basin from 2009-2020. Principal component analysis performed on 24 morphological traits suggest high overlap in morphological space, although *C. variegatus* had slightly thicker and deeper bodies than its native congener. Values of $\delta^{15}\text{N}$ were more enriched for *C. variegatus* suggesting their potential for feeding on different prey sources along the food chain. Stomach content analysis revealed some dietary overlap, in which both species consumed macroinvertebrate taxa mostly within the order Diptera. *Cyprinodon variegatus* is shown to be a more generalist feeder, consuming a variety of individual species within the order Diptera, while *C. rubrofluviatilis* exclusively consumed mosquito larvae (family Chironomidae, Diptera). Similarities in the morphology and feeding ecology between these two species could lead to ecological interactions such as competition for food resources, and perhaps hybridization between the native and invasive *Cyprinodon* species within the Brazos River. Our findings provide new insights for quantifying the ecological effects of invasive species in recipient populations.

INTRODUCTION

The introduction of invasive species is the second greatest threat to global biodiversity after habitat loss (Kerr 2014). An invasive species is a species that has been introduced outside its native range and is associated with ecological harm. Invasive species can have devastating ecological impacts that range from the genetic level to disturbing the function of an entire ecosystem. One major impact that an invasive species can have on a native species is through the process of hybridization (Cucherousset & Olden 2011). Hybridization with nonnative species is one of the largest threats to freshwater fishes in the western United States (Echelle 1991, Wilde & Echelle 1992). Breeding with introduced species threatens native species by either limiting the reproductive success of native species by out competing them for mates or resources (e.g., hybrid vigor), or by assimilating endemic genomes until genetically pure native individuals no longer exist (Huxel 1999, Rosenfield et al. 2004). Pupfish species (Cyprinodontidae: *Cyprinodon*) in the western United States are particularly vulnerable to hybridization with introduced congeners as their native ranges often occur in isolation from other pupfish species (Echelle & Echelle 1994). Pupfish species that are morphologically divergent have been shown to have high levels of genetic compatibility and thus can bring on rapid genetic changes to native populations once introduced (Villwock 1982, Hubbs 1980).

The Sheepshead minnow (*Cyprinodon variegatus*) has been introduced into several freshwater basins within the state of Texas. *Cyprinodon variegatus* is a popular bait fish and is believed to have been introduced into freshwater systems throughout Texas via bait bucket releases (Echelle & Connor 1989, Rosenfield & Kodric-Brown 2003). Although it is native to coastal waters, *C. variegatus* is extremely resilient to temperature and salinity changes and can successfully inhabit freshwater ecosystems (Lee et al. 1980, Hubbs et al. 1991). Such physiological tolerances may favor *C. variegatus* to become established in new environments.

In Texas, *C. variegatus* was introduced into several freshwater systems. These introductions have had devastating effects on its normally isolated native congeners. Within the Pecos River, *C. variegatus* was introduced sometime between 1980 and 1985, where it encountered the native cyprinodontid, the Pecos pupfish (*C. pecosensis*) (Echelle & Connor 1989, Rosenfield & Kodric-Brown 2003). Soon after introduction, hybridization was documented between *C. variegatus* and *C. pecosensis* (Echelle & Connor 1989). Five years after

introduction, these hybrids were estimated to have replaced the native population of *C. pecosensis* across more than half of its native range (Echelle & Connor 1989, Wilde & Echelle 1992). In the 1970s, *C. variegatus* was reported in Leon Creek, an isolated spring of the Pecos River. Two years after introduction, hybridization was observed between *C. variegatus* and the native Leon Spring pupfish (*Cyprinodon bovinus*) (Hubbs et al. 1978). Hubbs et al. (1991) report that *C. bovinus* nearly became extinct due to hybridization with the introduced congener, *C. variegatus*.

Although *C. variegatus* has been shown to negatively impact its native congeners within Texas, it has also been shown to coexist with them. Another introduction event occurred in the 1960s, where *C. variegatus* was accidentally introduced into Lake Balmorhea, an artificial impoundment that is home to the endangered Comanche Spring pupfish (*Cyprinodon elegans*) (Stevenson & Buchanan 1973). Although hybridization was observed to occur between *C. elegans* and *C. variegatus*, it was happening infrequently (Stevenson & Buchanan 1973). This can be attributed to the fact that *C. elegans* inhabits the springs that feed into the lake, while *C. variegatus* is restricted to the lake itself. (Stevenson & Buchanan 1973). While hybridizations with the introduced *C. variegatus* have greatly impacted other native pupfish of the Pecos region (*C. pecosensis* and *C. bovinus*), *C. elegans* appears to have persisted within Lake Balmorhea likely due to the two species differing habitat preferences. These findings suggest that there is variation in the impact that *C. variegatus* can cause in native congeners, which can depend on the physical and ecological space it occupies in relation to its native congeners.

The most recent discovery of *C. variegatus* has been in the upper Brazos River near Possum Kingdom Lake during a 2011 drought (Mayes & Wilde 2015, Mayes et al. 2019). In addition to *C. variegatus*, hybridization between *C. variegatus* and the native Red River pupfish (*Cyprinodon rubrofluviatilis*) appears to be occurring (Mayes & Wilde 2015, Mayes et al. 2019). This indicates the possibility of the formation of a hybrid swarm that could potentially lead to the genetic swamping of the native *C. rubrofluviatilis* form as previously observed with *C. pecosensis* and *C. bovinus* (Hubbs et al. 1991, Mayes et al. 2019). However, the frequency of hybridization occurring between the two species has not been reported. Further research is needed to confirm the potential impact of the introduced *C. variegatus* and potential hybrid

Cyprinodon on the native *C. rubrofluvialtilis*, as documented in other river systems (Stevenson & Buchanan 1973, Hubbs et al. 1978, Echelle & Connor 1989).

In this study, we investigated the potential impacts of *C. variegatus* and hybrid individuals in the native population of *C. rubrofluvialtilis* in reaches of the middle and upper Brazos River, Texas. *Cyprinodon rubrofluvialtilis* is native to the Brazos River and upper Red River drainages within Texas (Ostrand & Wilde 2002). Currently, it is listed as threatened within the state of Texas, so monitoring the threats to its survival within the state is imperative. We used an integrative approach consistent of morphological, dietary, and isotope analyses to examine the ecological niche these cyprinodontids to make inferences in niche space occupied by the native, the invasive, and their potential hybrids. If an introduced species niche greatly overlaps with a native species, it has the potential to be more competitive than the native species at exploiting their shared resources (i.e., niche replacement hypothesis) (Ricciardi et al. 2013). Niche overlap between native, nonnative, and hybrid species can result in competitive displacement with subsequent impacts in fitness or even extinction of the native species (Gause 1934). Morphological analyses can be used to infer functional niche space of a species and help predict invasion success (Azzurro et al. 2014). Dietary analysis based on stomach contents analysis is used to estimate prey abundance and prey consumption (Bourke et al. 1999). Stomach contents analysis provides insight into what dietary resources invasive species are exploiting within recipient ecosystems (Vaughan et al. 2016). Stable isotope analysis is based on ratios of carbon [$^{13}\text{C}/^{12}\text{C}$] and nitrogen [$^{15}\text{N}/^{14}\text{N}$] and is used to estimate the isotopic niche space of invasive species (Vander Zanden et al. 1999). $\delta^{13}\text{C}$ value is assumed to reflect how material from various production sources is assimilated into consumer tissue; $\delta^{15}\text{N}$ reflects vertical trophic position of consumer (Montaña et al. 2020). Stable isotope analysis can be used to reconstruct diets and assign trophic positions to species (Boecklen et al. 2011). A combination of these methods will be used to give a clear picture of the niche each species occupies. We predict that both *C. variegatus* and hybrid individuals will occupy morphological, dietary, and isotopic niche spaces similar to *C. rubrofluvialtilis*. The potential overlap in ecological niche space with *C. variegatus* and the hybrid *Cyprinodon* species could indicate a serious threat to the continued existence of *C. rubrofluvialtilis* within the Brazos River.

METHODS

Fish Surveys

Surveys for cyprinodontids were conducted by Gene Wilde of Texas Tech University from 2009-2017 along 13 sites within the upper Brazos River (Figure 1). Fish were collected using a seine net and were preserved in 70% EtOH. All fishes were deposited at SFASU fish collection for analysis in this project. Fish samples were sorted by site and date of collection and then identified down to the lowest taxonomic level of species using taxonomic key by Hubbs et al. (1991). Fishes were classified based on the following traits: body shape and stomach scaling (Hubbs et al. 1991). *Cyprinodon rubrofluvaitilis* had longer a body, rectangular bars, and naked stomachs. *Cyprinodon variegatus* had a deeper body, triangular bars, and a fully scaled stomach. Individuals that deviated from either patterns and had an intermediate morph form was labelled as a potential hybrid and analyzed as such.

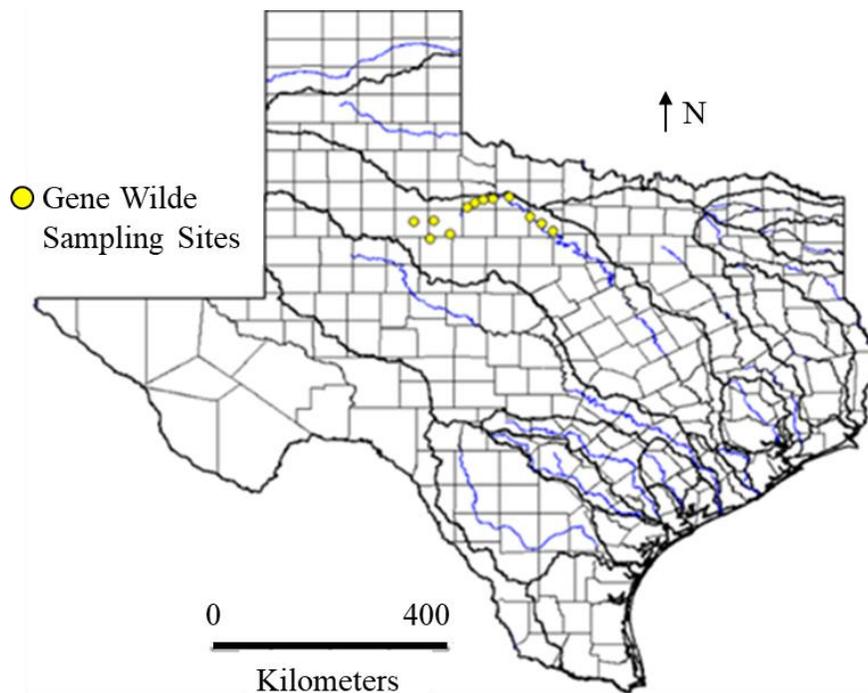


Figure 1. Map showing 13 sites sampled by Gene Wilde of Texas Tech University from 2009-2017 along the upper Brazos River, Texas.

Morphological Measurements

Morphological measurements were taken from 10 individuals per species per site when available. Measurements were taken using a caliper (precision=0.01mm) in a standardized manner. In total, 25 morphological traits associated with foraging ability and habitat use were measured (Montaña et al. 2020, Montaña & Winemiller 2013). Descriptors of body shape and fin size will be converted to proportions following the protocol outlined by Montaña & Winemiller (2013) to remove influences of body size. Ratios of body size can introduce allometric bias into shape analysis. However, when using single class of specimens, allometric influence should be negligible to interpret interspecific morphological comparisons (Winemiller 1990, Montaña & Winemiller 2013). Linear morphological distances were transformed into ratios following the protocol outlined by Montaña & Winemiller (2013). Body width was used as the denominator for mouth width. Head length was used as the denominator for the ratio of eye diameter, snout length, and snout-mouth protrusion. Head depth was used as the denominator for eye position. Standard length (SL) was used as the denominator for all other traits excluding those listed previously. These values were then log transformed and used in a principal components analysis (PCA). PCA was used to describe the functional trait space of the native, nonnative and hybrid species (Montaña et al. 2020, Schalk et al. 2018, Azzurro et al. 2014). Permutational multivariate analysis of variance (PERMANOVA) with 9999 unrestricted permutations was performed to test whether fish species significantly different in morphospaces. If the PERMANOVA result was significant, post-hoc pairwise tests were conducted to compare differences.

Stomach Contents Analysis

Dietary analysis via stomach contents analysis was used to determine the abundance and type of prey consumed by individual species. Intestines were removed and dissected under a microscope to assess stomach contents. Prey items consumed were identified to the lowest taxonomic level possible using taxonomic keys. Both volume and abundance of prey items consumed were recorded. To assess whether native and nonnative species exhibit convergent patterns in dietary niches, we estimated the volumetric proportions of food items used by each species (Montaña & Winemiller 2013). Volumetric proportions were estimated as described by Winemiller (1990). Food items were then be placed into major taxonomic orders such as: Diptera, Hemiptera, Odonata, Ephemeroptera, Coleoptera, Trichoptera, and any additional order

represented in the stomach contents. Proportions of these diet items were then compared across all species to assess potential convergent patterns.

Stable Isotope Analysis

Stable isotope analysis was used in combination with stomach contents analysis to determine resource overlap between congener species. Stable isotope analysis was prepared following the protocols by Arrington & Winemiller (2002). Due to their small size, the entire specimen (minus the gut) was processed for stable isotope analysis. Prior to analysis, all individuals preserved for SIA were soaked in deionized water for 60 minutes to remove any excess salt, mineral, or dirt on their tissues. Once rinsed, fish was dissected using sterile scalpel and tweezers. Their gut contents were removed and placed into a separate labelled vial containing ethanol for further analysis. The individual was then be placed into a drying oven at 60°C for 48 hours. After 48 hours, the dried fish was ground into a fine powder using a sterile mortar and pestle. For each fish sample, a subsample 0.20-0.25g of powder were weighed and packaged into an Ultra-Pure tin capsule. Each pellet was given a corresponding code based on the fish's standard length and collection date/location. Samples were sent to the Analytical Chemistry Laboratory, Institute of Ecology, at the University of Georgia for analysis on stable carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotope signatures using an isotope ratio spectrometer. Biplots of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were made to compare isotopic niche spaces of native, nonnative, and hybrid fish species at sites where all species occur.

RESULTS AND DISCUSSION

Abundance Data

Fish species were collected from 15 sites along the upper Brazos River from 2009-2017. Abundance data were calculated for each species per year (Figure 2). *Cyprinodon rubrofluviatilis* was consistently collected during all 9 sampling years, which is as expected as the endemic species. *Cyprinodon rubrofluviatilis* abundance remained consistent from 2009-2011, with a gradual increase in 2012 and a peak collected of 1366 individuals in 2013 (Figure 2). After 2013, there was a progressive decline in individuals collected, with only 291 individuals having been collected in 2017 (Figure 2). *Cyprinodon variegatus* was first reported to occur in the Brazos River in 2011 near Possum Kingdom Lake (Mayes & Wilde 2015, Mayes et al. 2019).

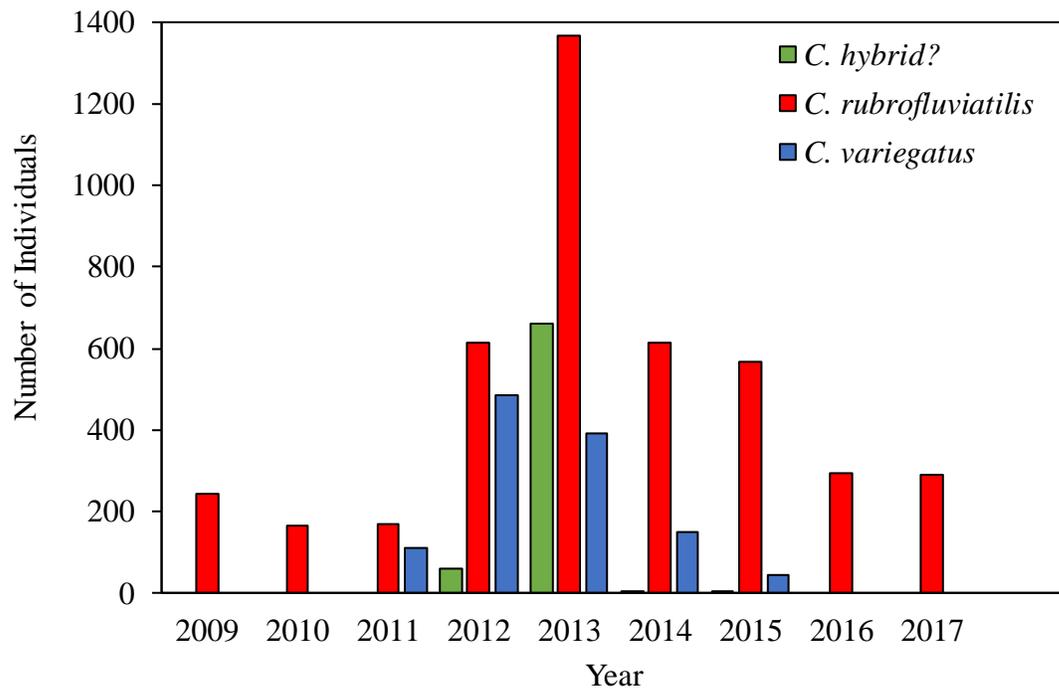


Figure 2. Abundance of three *Cyprinodon* species collected in the Brazos River, Texas, between 2009 and 2017 by Gene Wilde at Texas Tech University.

Abundance data showed that *C. variegatus* was first collected in 2011, its abundance peaked in 2012 with 483 individuals being collected in the upper Brazos River. After 2012, abundance of *C. variegatus* decreased gradually from 2013-2015, no individuals were collected in 2016 or 2017 (Figure 2). The first year that potential hybrid individuals were shown to occur was in 2012, one year after the introduction of *C. variegatus*. Assumed hybrid individuals reached peak abundance in 2013 with 661 individuals collected. Following this peak there was a drastic decline in the number of potential hybrids collected, no hybrid individuals were collected from 2016-2017 (Figure 2).

Cyprinodon variegatus was believed to have introduced into the Brazos River into Possum Kingdom Lake in 2011 and this introduction was presumably due to a bait bucket release (Mayes & Wilde 2015, Mayes et al. 2019). The introduction of this *C. variegatus* happen to coincide with the worst drought Texas had experienced since 1956 (Nielsen-Gammon 2011). Beginning in March 2011, Texas experienced record low rainfall and record high summer temperatures (Nielsen-Gammon 2011). This severe drought impacted the flow of rivers throughout the state, including the Brazos River. Flow data from above and below Possum Kingdom Lake shows low discharge starting in 2011 and continuing through 2014 (Figure 3). After introduction in 2011 *C. variegatus* seems to have been able to successfully establish a

population and begin to hybridize as it did in other basins within the state (Echelle & Connor 1989, Hubbs et al. 1978). Decline of *C. variegatus* and assumed hybrids is correlated with the increased flow to of the Brazos River starting in 2015 (Figure 3f). Upstream of Possum Kingdom in Young County near locations where *C. variegatus* and hybrids were collected, discharge was shown to increase from 51.2 ft³/sec on average to 890 ft³/sec in 2015 and then 1242 ft³/sec in 2016.

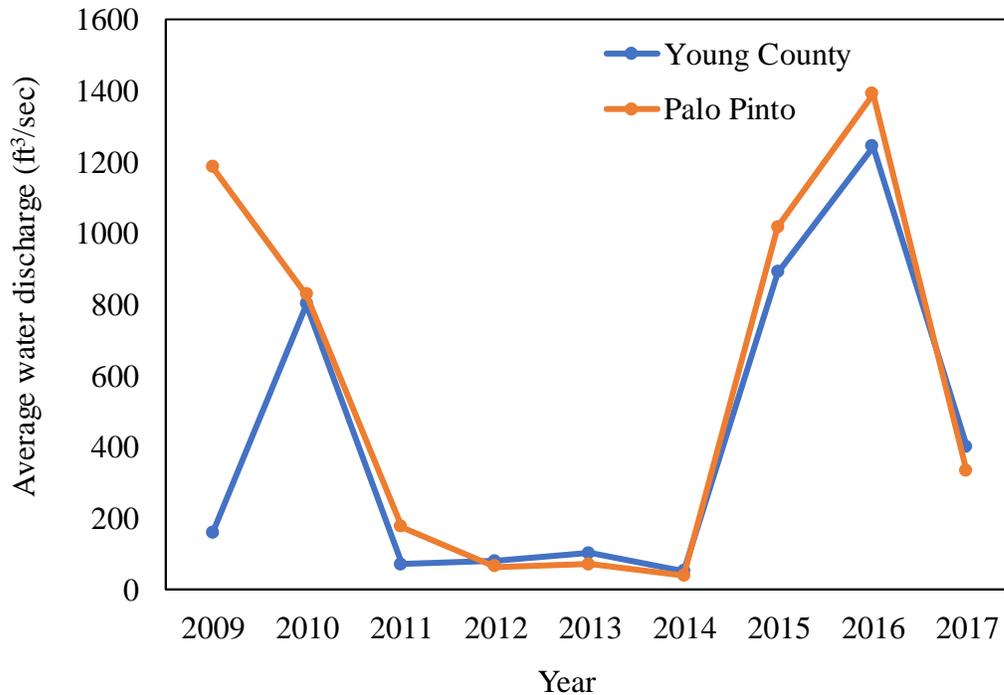


Figure 3. Water discharge data (ft³/sec) for two sites along the Brazos River above (Young County, Texas) and below (Palo Pinto County, Texas) Possum Kingdom Lake from 2009-2011. Data was obtained from the U.S. Geological Survey National Stream Water-Quality Monitoring Networks (WQN) and the Brazos River Authority.

Cyprinodon variegatus is native to coastal waters and has been reported to prefer relatively shallow water with little current or wave action present (Johnson 1980). While *C. variegatus* is tolerant of extreme changes in temperature and salinity, it may not have been tolerant to increased flow (Kilby 1955, Simpson & Gunter 1956). *Cyprinodon rubrofluviatilis* is endemic to the Brazos and is evolved to tolerate the changes in flow associated with river systems. Both *C. variegatus* and potential *C. hybrid*? were assumed to be extirpated from the Brazos River in 2016 because of the increased average flow; however, further research is needed to investigate *C. variegatus* and hybrid individual's ability to survive during high flow events.

Functional Traits

A total of 372 individuals were measured for 25 morphological traits (*C. hybrid?* [n=50], *C. rubrofluvialtilis* [n=248], *C. variegatus* [n=74]). *Cyprinodon* species assemblages were shown to occupy different functional niche spaces (PERMANOVA, $F=19.961$, $p<0.001$). The PCA resulted in two main axes that explained 39.07% of the total morphological variation (PC1=23.85%, PC2=15.22%, Figure 4). The first xx-axis (PC1) was primarily associated with a body depth gradient (Figure 4). Positive PC1 loadings were associated with individuals having deeper bodies and wider peduncles (e.g., *C. variegatus*), while negative PC1 loadings were associated with longer heads and longer peduncles (e.g., *C. rubrofluvialtilis* and *C. hybrid?*) (Figure 4). PC2 was primarily associated with a trophic ecology gradient (Figure 4). Positive PC2 loadings were associated with individuals having larger gape width and smaller fins, while negative PC2 loadings were associated with longer snout-mouth protrusions. Results from PERMANOVA post-hoc pairwise comparisons revealed significant differences in functional trait

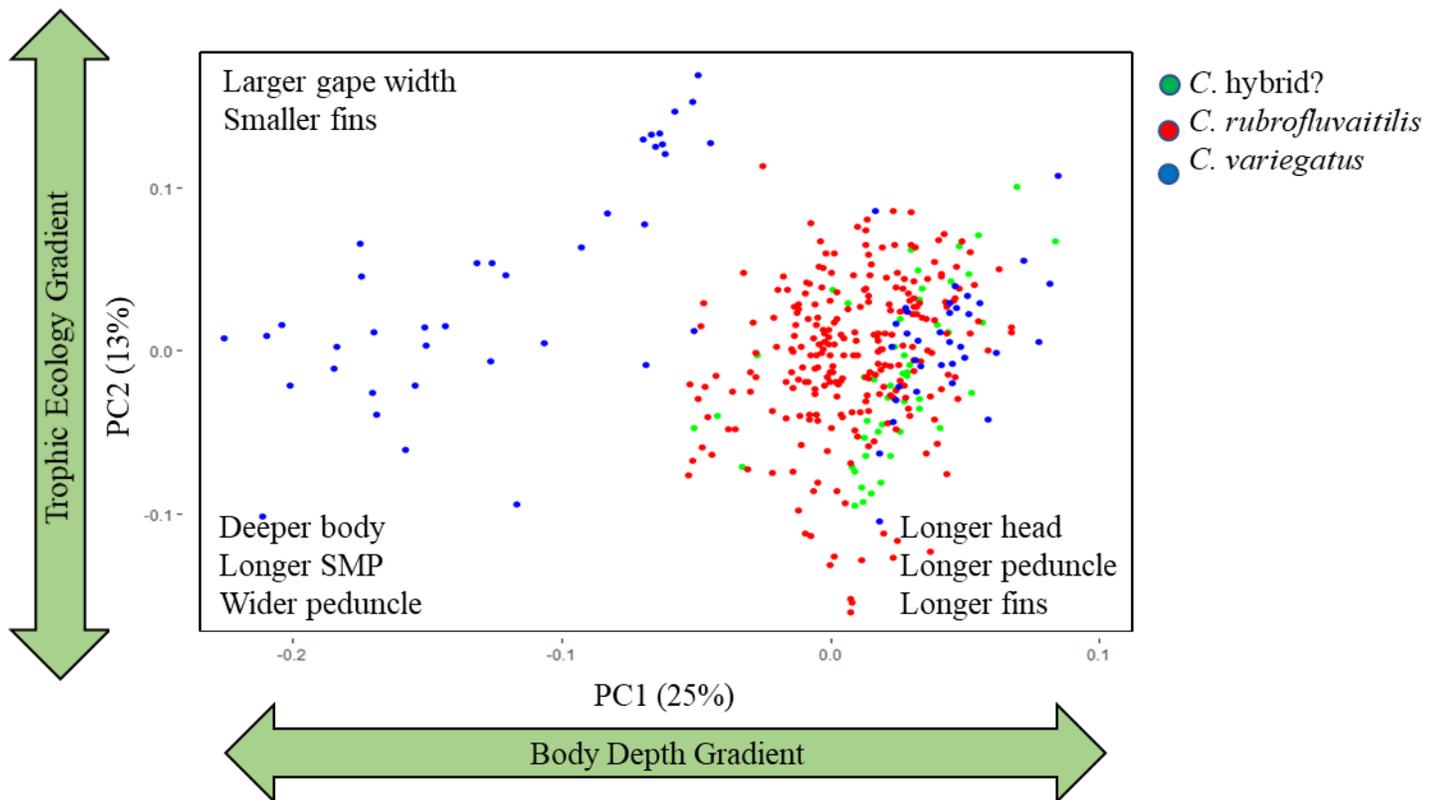


Figure 4. Principal Component Analysis (PCA) showing the morphological niche space occupied by each species (*C. hybrid?* n=50, *C. rubrofluvialtilis* n=248, *C. variegatus* n=74). PCA was done using 23 morphological traits associated with foraging ability and habitat use. Fish were collected from 2011-2017.

space occupied by *C. variegatus* and *C. rubrofluviatilis* ($F=26.32, p<0.001$), *C. rubrofluviatilis* and *C. hybrid?* ($F=15.21, p<0.00$), and *C. variegatus* and *C. hybrid?* ($F=12.40, p<0.001$).

While *Cyprinodon* species appear to occupy different morphospaces, there is still a large degree of overlap between species. For example, *C. variegatus* has slightly deeper bodies, but there was evidence of overlap in body shape with *C. rubrofluviatilis* (Figure 4). The largest degree of overlap was seen between *C. rubrofluviatilis* and the potential *C. hybrid?*, which seem to be highly associated with longer heads and longer bodies (Figure 4). Functional traits measured in this study were assumed to reflect species foraging ability and habitat use (Montaña et al. 2020, Montaña & Winemiller 2013). Overlap in functional trait space between native and invasive species of *Cyprinodon* have been inferred previously. Echelle et al. (1972) concluded that interspecific interactions were the primary factors limiting the distribution of *C. rubrofluviatilis* within the Brazos River. Prior to the introduction of *C. variegatus*, *C. rubrofluviatilis* did not encounter any other *Cyprinodon* species within the Brazos River. The introduction of a nonnative congener could lead to severe interspecific competition between the two species likely due to shared morphological and ecological similarities, thus limiting the range of *C. rubrofluviatilis*. Our morphological analysis revealed overlapping in the morphological and functional niche among native, invasive and hybrid species. This overlap implies the potential for competition for resources (e.g., food, space), which could have devastating impacts on the native species fitness and continued persistence within the Brazos River.

The functional traits measured are assumed to reflect the species fundamental niche, but novel environments nonnative species experience could result in a different realized niche. When combined, both dietary and isotopic analyses give a more complete picture of how an invasive species interacts with others within their recipient communities. Results from dietary and isotopic analysis, in combination with the results from morphological analyses better reflect *C. variegatus* and *C. hybrids?* niches within the Brazos River.

Dietary niche partitioning

Overall, a total of 23 different food items were identified in the stomach contents of the 3 different *Cyprinodon* species. Food items consisted of organic detritus, algae, sand/gravel, 2 orders within the Phylum Arthropoda. For *C. rubrofluviatilis*, the most abundant items consumed

by volume were organic detritus (49.79%), followed by sand/gravel (35.89%), algae (7.94%), and Diptera (5.03%) (Figure 5). For *C. variegatus*, the most abundant items consumed by volume were organic detritus (74.12%), followed by algae (8.13%), sand/gravel (8%), Diptera (6.66%), and Ostracoda (1.23%). For the assumed hybrid assemblage, the most abundant items consumed by volume were organic detritus (51.09%), sand/gravel (36.51%), algae (6.19%), and Diptera (4.96%). Other food items were present in the stomach contents for each species but were rarely found and made up <1.0% of the stomach contents by volume. Overall, the three species show similar patterns in food items observed in stomach contents. *Cyprinodon rubrofluviatilis* and assumed hybrids shared almost identical food preferences based on their volumetric food item portions. *Cyprinodon variegatus* also showed similar patterns in food items consumed, but stomach contents contained a higher portion of organic detritus and a lower portion of sand/gravel (Figure 5). *Cyprinodon variegatus* also consumed a large amount of Ostracoda (small crustaceans within the Phylum Arthropoda), neither of the other two species was seen to consume any prey items within this class of organisms.

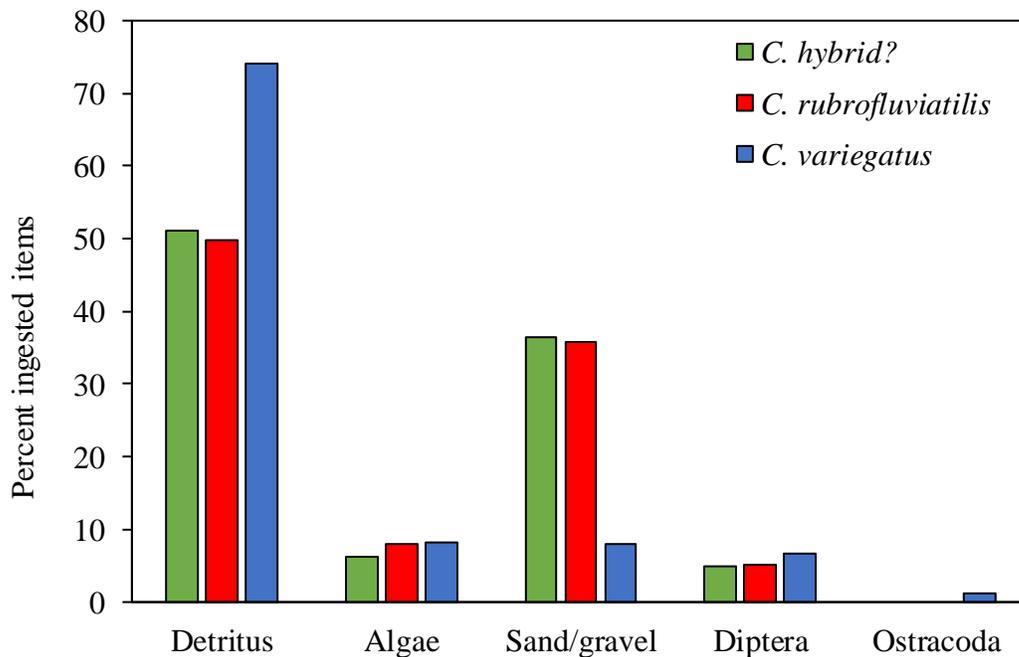


Figure 5. Percentage of food items identified in stomach contents analysis by species (*C. hybrid?* n=40, *C. rubrofluviatilis* n=90, *C. variegatus* n=73). Only food items consisting of >1.0% of stomach contents included in the graph. Individuals sampled from 2012-2014.

We observed some variation of families consumed within the order Diptera (i.e., flies). All three species were shown to consume high portions of prey items within the order Diptera, however *C. variegatus* consumed a larger variety of individuals representing other families than either of the other *Cyprinodon* species. *Cyprinodon variegatus* consumed items from 5 different families in the order Diptera: Chironomidae (85.94%), Thaumaleidae (2.41%), Dolichopodidae (4.02%), Tanyderidae (1.21%), and Tanypodindae (6.43%) (Figure 6). *Cyprinodon rubrofluviatilis* consumed prey items from 2 families within the order Diptera: Chironomidae (99.38%) and Dixidae (0.62%), while assumed hybrids were shown to consume 1 family exclusively, Chironomidae (Figure 6).

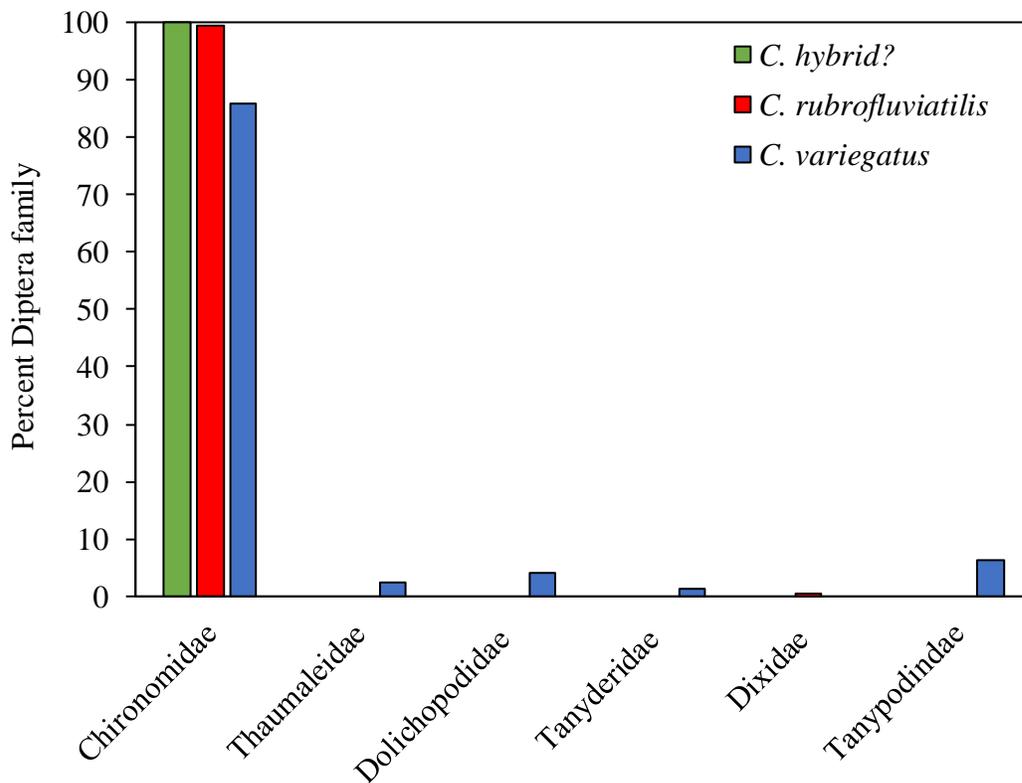


Figure 6. Percentage of families within the Order Diptera consumed by species (*C. hybrid?* n=40, *C. rubrofluviatilis* n=90, *C. variegatus* n=73). Individuals sampled from 2012-2014.

Our findings agree with previous research conducted on both *C. rubrofluviatilis* and *C. variegatus* (Echelle 1973, Miller & Robison 2004, Doherty 1987). *Cyprinodon rubrofluviatilis* has been shown to be an intensely active, bottom-feeding omnivore that feeds mostly on midge (Diptera) larvae (Echelle 1973, Miller & Robison 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. 1972a). This feeding behavior suggests that individuals accidentally ingest large amount of substrate such as sand and gravel. We observed large portions of substrate in the stomach contents of *C. rubrofluvialtilis*, confirming this feeding behavior. *Cyprinodon variegatus* is also shown to be an omnivorous fish, and is reported to feed largely on organic detritus, algae, microcrustaceans, and dipteran larvae (Doherty 1987, Harrington & Harrington 1961) *Cyprinodon variegatus* has also been shown to display “plowing” behaviors as it picks the substrate for food items, similar to *C. rubrofluvialtilis* (Foster 1967). Little is known about the feeding habitats of hybrid *C. rubrofluvialtilis* and *C. variegatus* offspring. Our data suggests that *C. hybrid* and *C. rubrofluvialtilis* have almost identical patterns in stomach contents. While *C. variegatus* diet does differ slightly, there is still a large degree of overlap with the native species. This overlap in dietary niche indicates that *C. rubrofluvialtilis* is competing for food resources with both the invasive and hybrid species. This could have devastating impacts on the fitness of the native species if *C. variegatus* and hybrid populations continue to persist in the Brazos River.

Isotope niche partitioning

Tissues from 142 individuals were used in isotopic analyses (*C. hybrid* [n=40], *C. rubrofluvialtilis* [n=27], and *C. variegatus* [n=75]). Biplots $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were made to show overlap in the isotopic niche space occupied each species (Figure 7). Overall, all three *Cyprinodon* species showed similar patterns in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values fixed in tissue samples. There were two clusters of *C. variegatus* that varied in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures (Figure 7). A group of 12 *C. variegatus* were enriched in nitrogen as all other individuals, but more depleted in carbon. Stable carbon isotopes are known to reflect the primary carbon sources within a food web (Jackson et al. 2011). Another group of 10 *C. variegatus* were enriched in carbon as the majority of other individuals but were more enriched in nitrogen. Stable nitrogen isotopes reflect the vertical trophic position of the consumer (Jackson et al. 2011). To better understand these differences, a regression analysis was performed to see if variation in $\delta^{15}\text{N}$ signatures was correlated with standard length (Figure 8). Larger individuals may have been feeding higher up in the food chain, and thus would be more enriched in nitrogen. *Cyprinodon variegatus* $\delta^{15}\text{N}$ are not strongly correlated with standard length ($y=0.74x-9.33$, $R^2=0.30$) (Figure 8). We did find that these separated groups were directly correlated with one site at Graham, Texas, and the year that

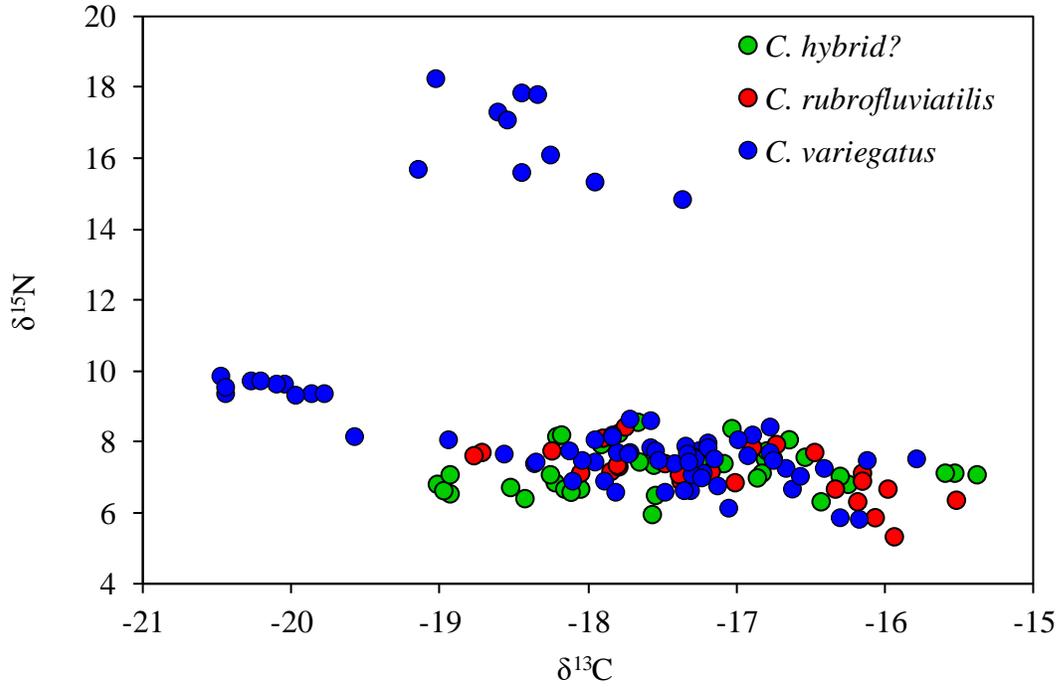


Figure 7. Biplots of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures fixed in fish tissue samples (*C. hybrid?* n=40, *C. rubrofluviatilis* n=27, *C. variegatus* n=74) showing the isotopic niche space occupied by each species. Individuals sampled from 2012-2014.

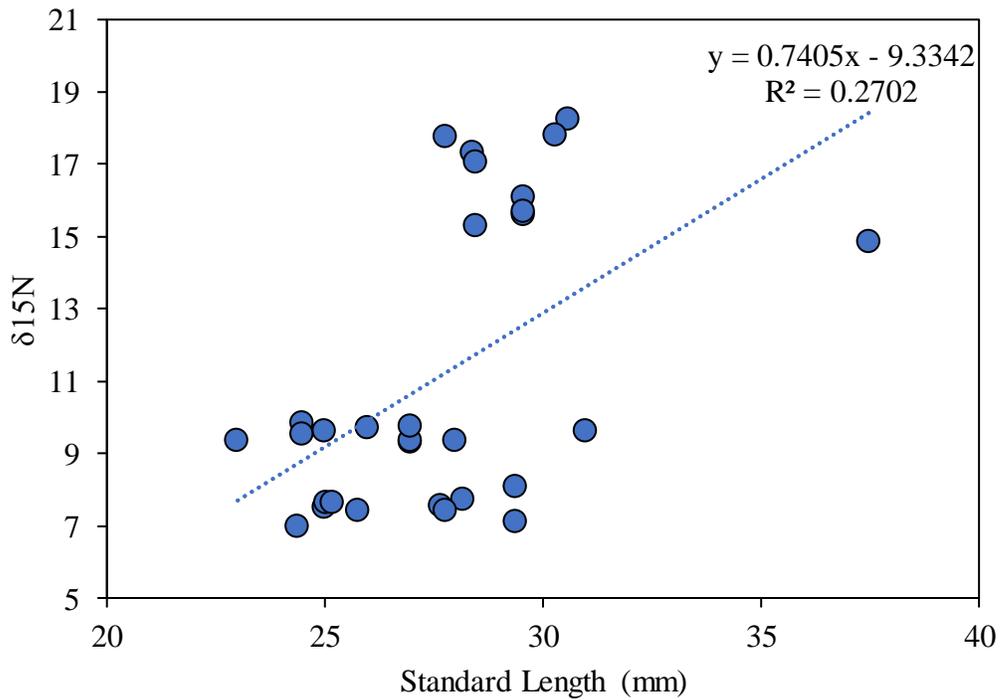


Figure 8. Regression analysis between Nitrogen ($\delta^{15}\text{N}$) and body size (standard length, mm) of *Cyprinodon variegatus* (n=22).

nitrogen, and individuals collected at Graham in 2014 were more depleted in carbon. Variation in stable carbon and nitrogen isotopes can reflect pollution in aquatic environments (Grossowicz et al. 2019). We do not have data on basal resources or water quality at the site, but this variation is possibly due to pollution or other site conditions affecting the basal resources (Grossowicz et al. 2019). Although this is just speculation based on our knowledge of basal resources and pollution, future samplings of these species along the Brazos River should be accompanied but basal resource collection.

Barring these two groups of *C. variegatus* from Graham, TX from 2013 and 2014, all three *Cyprinodon* species were similarly enriched in carbon and nitrogen. Similarities in carbon enrichment suggests that species were feeding on within the same food chain (Jackson et al. 2011). Basal resources were not collected at the site; therefore, we cannot make inferences in what carbon resource the species were utilizing. Similarities in nitrogen enrichment suggests that species were feeding on the same trophic level as well (Jackson et al. 2011). Stomach contents analysis suggests that they were feeding relatively low on the food chain. All species feed primarily on detritus, algae, and dipteran larvae (Figure 3). This large degree of overlap in isotopic niche space reaffirms patterns observed in stomach contents and morphological data.

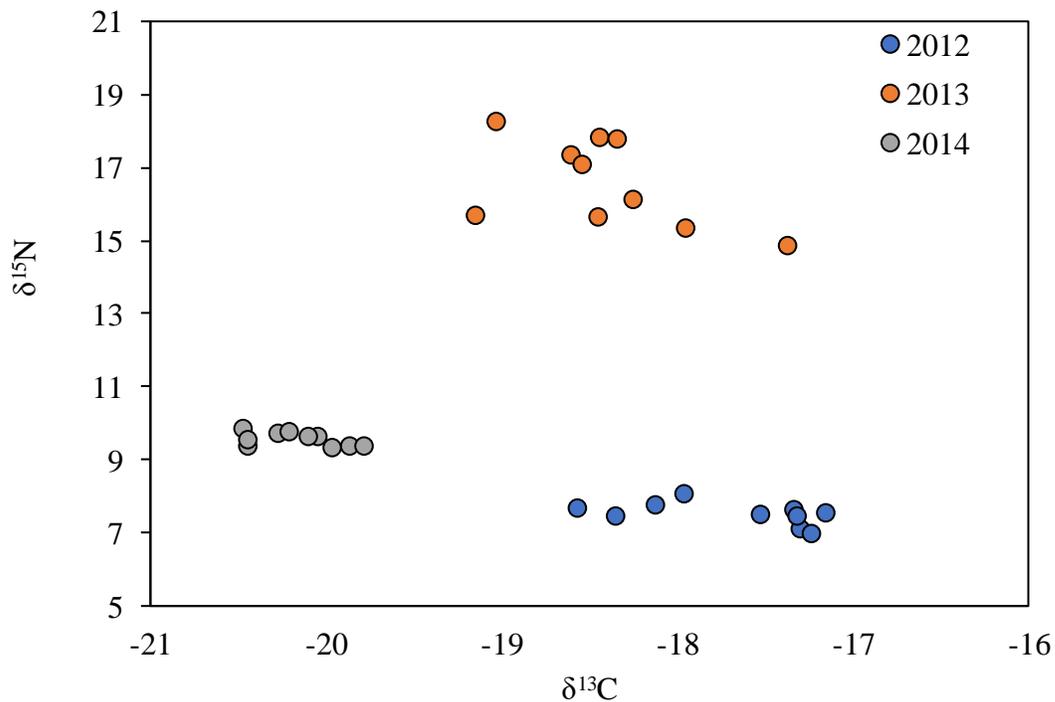


Figure 9. Biplot of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of *Cyprinodon variegatus* (n=32) collected at sites in Graham, Texas during 2012, 2013, and 2014.

CONCLUSION

The invasive species, *Cyprinodon variegatus*, and its hybrid offspring show similar morphological, dietary, and isotopic niches as the native *Cyprinodon rubrofluviatilis*. The invasive pupfish species has been shown to have devastating ecological impacts on native pupfishes throughout the state of Texas. Mainly through the process of hybridization, *Cyprinodon variegatus* has been shown to bring on rapid genetic changes to native populations once introduced (Hubbs 1980). With its recent introduction into the Brazos River in 2011, *Cyprinodon variegatus* has been observed to hybridize with the endemic pupfish species. This invasive species represents a serious threat to the survival of the endemic *Cyprinodon rubrofluviatilis*. Abundance of *Cyprinodon variegatus* within the Brazos River has declined in recent years, but it has still been found in contemporary survives (2020-2021) in low numbers. Pathways of introduction of *Cyprinodon variegatus* need to be investigated and closed to prevent future introductions throughout the state. Prevention of these introductions are the best management strategy to ensure the persistence of *Cyprinodon rubrofluviatilis* within the Brazos River.

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