FITNESS OF INTERSPECIFIC HYBRIDS IN THE GENUS CYPRINELLA: AN EVALUATION OF SWIMMING PERFORMANCE IN NORTH AMERICAN STREAM FISHES

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BY

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DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES IN FULFILMENT FOR THE DEGREE OF MASTER OF SCIENCE AT GEORGIA COLLEGE & STATE UNIVERSITY

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Fitness of Interspecific Hybrids in the Genus Cyprinella: An Evaluation of Swimming Performance in North American Stream Fishes

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PREFACE

This thesis has been written in journal format and conforms to the style appropriate to my discipline. This manuscript will be submitted for publication in the Journal of Fish Biology, a peer reviewed interdisciplinary scientific journal, and therefore reflects the required formatting for this publication. This thesis does not contain a list of tables or figures since these are not included in the submission guidelines for contributors to this journal. Tables and figures follow the text of the manuscript as required by the Journal of Fish Biology and this thesis committee.
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ABSTRACT

Due to anthropogenic pressures, freshwater ecosystems are being rapidly destroyed worldwide. Accordingly, human impacts have also resulted in the loss of native aquatic biodiversity. In particular, the introduction of non-native species to aquatic habitats is of great concern. Historically, the introductions of many non-native freshwater fishes are the result of commercial baitfish aquaculture, food industries, and private aquarium release. The red shiner (Cyprinella lutrensis) is a small stream fish that is endemic to the Central U.S., and its natural range is restricted to East of the Mississippi River. Since the 1950’s, red shiner have been commercially raised and transported across the globe as fishing bait and ornamental fish. During the early 1990’s populations of invasive red shiner were first observed in the Coosa River Basin, located in Northwest Georgia, USA. Originating from bait bucket releases, the invasive red shiner quickly established within the Upper Coosa River Basin (USA) and now hybridizes with at least one native species of Cyprinella, the blacktail shiner (C. venusta). Over the past thirty years, red x blacktail shiner hybrids have become increasingly abundant and demonstrate an unknown level of hybrid viability. To date, little is known about the relative fitness of red x blacktail shiner hybrids compared to their parental species. If hybrids exhibit equal or greater fitness compared to parental species, then this could result in regional extinctions of native Cyprinella species. To assess the relative fitness of hybrid shiner, we evaluated morphology and maximum swimming velocity for red, blacktail, and hybrid shiner. Our results suggest that hybrid shiner are intermediate to parental species in both swimming performance and body morphology. In conclusion, we propose that red shiner x blacktail shiner hybrids may have a competitive level of fitness when compared to parental species, and hybrid shiner are potentially more harmful to native Cyprinella than pure red shiner.
INTRODUCTION

Over the past century, rapid advancements in transportation and trade have negatively impacted global biodiversity. Of all the ecosystems on Earth, freshwater habitats are particularly sensitive to species loss (Ricciardi and Rasmussen, 1999). Anthropogenic threats to freshwater biodiversity are diverse, however habitat disturbance and the introduction of non-native species are of the greatest concerns (Sala et al., 2000). Evolutionarily, there are many prezygotic and postzygotic barriers that keep native and non-native species distinct. Unfortunately, humans have disrupted these barriers through a variety of intentional and unintentional activities (Kolar and Lodge, 2000). Such activities include the intentional transport and commerce of living organisms and accidental “hitchhiking” events from human movement across the globe (Lockwood et al., 2013). Ultimately, these activities have resulted in the introduction and spread of countless non-native freshwater species that cost countries billions of dollars per year (Pimentel et al., 2005).

Globally, fishes are economically valuable and are often introduced to new regions of the world to meet societal demands. It is estimated that 51% of fish transport is for food aquaculture, 21% for ornamental hobby, 12% for sport fishing, and 7% for wild fisheries (Gozlan, 2008). Collectively, fishes are among the most introduced vertebrates in the world, totaling an estimated 624 species (Gozlan, 2008). Furthermore, natural resource agencies often introduce non-native game fishes to lakes and rivers to meet the desires of recreational anglers (Kolar and Lodge, 2000; Rahel 2002; Pelicice at al., 2014). These introductions include fishes such as rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), and brook trout (*Salvelinus fontinalis*) as well as largemouth bass (*Micropterus salmoides*) (Gozlan et al., 2010). Similarly, many species of exotic fishes are introduced by recreational anglers discarding unused bait (Kilian et al., 2012), and tropical fishes are frequently released to novel habitats through the
aquarium fish trade (Padilla and Williams, 2004). Consequently, native fish communities have substantially changed due to species loss resulting from the introduction of non-native species (Helfman, 2007). Fishes are one of the most threatened groups of vertebrates on Earth, with 1,344 species classified as vulnerable in 2022 (IUCN, 2022).

In the United States (U.S.) alone, there are an estimated 138 species of introduced, non-native fishes (Courtenay et al., 1991; Courtenay, 1993, 1997). Historically, most cases of successful establishment have occurred in areas of the U.S. with mild climates such as Florida (fifty species), California (fifty-six species), and Hawaii (thirty-three species), (Courtenay, 1997; Dill & Cordone, 1997; Maciolek, 1984). As suggested by Wilcove and Bean (1994), forty-four species of fishes endemic to the U.S. are now listed as endangered due to the introduction of non-native fishes, and an additional twenty-seven native species are threatened. For example, introduced mosquito fish (*Gambusia affinis*) have been responsible for the decline of at least fifteen native fish populations in the desert rivers and streams of the Southwestern U.S. (Schoener, 1981). Similarly, since 1963 Asian grass carp (*Ctenopharyngodon idella*) have spread across thirty-five states in countless attempts to control aquatic weeds (Haughton, 1978; Guillory and Gasaway, 1978). In addition to habitat modification caused by invasive grass carp, it is also believed that introduced grass carp were the carrier of Asian tapeworms (*Bothriocephalus acheilognathi*) into the U.S. (McCann, 1984; Macdonald, 1989). As a result, Asian tapeworms are now commonly found in many species of North American cyprinids and temperate basses (Marcogliese, 2008; Choudhury et al., 2006).

One lesser known, yet harmful species of invasive freshwater fish is the red shiner (*Cyprinella lutrensis*). Red shiner are native to the Central United States, including the Mississippi River Basin from Southern Wisconsin to South Dakota, Louisiana, Texas, New
Mexico, and Colorado (Page and Burr, 2011). Over the last fifty years, red shiner have been introduced to many states outside of its native range (Fuller, 1999; Walters et al., 2008; Blum et al., 2010; Ward et al., 2012; Glotzbecker et al., 2016 B). Through bait-bucket and aquarium releases, populations of non-native red shiner can now be found in California, Arizona, Utah, Georgia, and Virginia (Fuller et al., 1999). During the 1970’s red shiner were introduced to at least five drainage basins in the Southeastern United States (Fuller et al., 1999). In recent research by Glotzbecker et al. (2016 A), the invasion history of red shiner across the U.S was reconstructed by analyzing the phylogenetic relationships of native and non-native populations. Mitochondrial analysis revealed four distinct lineages of red shiner found in the U.S. and demographic modeling suggests multiple, independent introduction events across the U.S. (Glotzbecker et al., 2016 A).

A harmful characteristic of introduced red shiner is their ability to successfully hybridize with endemic *Cyprinella*, such as the blacktail shiner (*Cyprinella venusta*) (Walters et al., 2008; Glotzbecker et al., 2016 B). As early as the 1950’s ichthyologists began to observe naturally occurring red shiner x blacktail shiner hybrids in turbid streams of Southeastern Texas (Hubbs and Hobbs, 1953). Similarly, Walters et al. (2008), began investigating invasive red shiner populations located in the Upper Coosa River Basin (UCRB) of Northern Georgia and Alabama, USA. Phenotypic and genetic analyses revealed that invasive red shiner were rapidly hybridizing with native blacktail shiner throughout much of the UCRB (Walters et al., 2008; Blum et al., 2010; Glotzbecker et al., 2016 B). As presented in Glotzbecker et al. (2015), it is suggested that habitat disturbance and elevated water turbidity reduces prezygotic reproductive isolation and facilitates hybridization between the two species by masking visual signals. From a conservation perspective, hybridization between native and non-native fishes can be harmful, leading to the
loss of native biodiversity through species collapse (Seehausen et al., 1997, 2008; Seehausen, 2006; Taylor et al., 2006; Glotzbecker et al., 2015).

In areas with extensive hybridization, hybrid zones can rapidly form (Seehausen, 2006). Hybrid zones are areas where two genetically distinct populations come into contact and hybridization occurs (Allendorf et al., 2001). Within these areas, hybrid swarms can develop when a population mostly contains hybrid individuals that have resulted from backcrossing with parentals and then mating with other hybrids (Seehausen, 2006). When hybrid swarms form after an introduction event, it can lead to a decrease in the biodiversity of native taxa (Huxel, 1999; Epifanio and Philipp, 2000; Hall et al., 2006; Ward et al., 2012). After three-decades of contact between invasive red shiner and native blacktail shiner, previous studies have identified the formation of a red shiner x blacktail shiner hybrid swarm in the UCRB (Walters et al., 2007; Blum et al., 2010; Glotzbecker et al., 2015, 2016). Repeated field sampling over the course of seven years suggests that the hybrid swarm is rapidly expanding both upstream and downstream of Weiss Lake (Alabama, USA), into the mainstem and tributaries of the Upper Coosa River (Walters et al., 2008; Blum, 2010; Ward et al., 2012; Glotzbecker et al., 2015, 2016). The expansion of the red shiner x blacktail shiner hybrid swarm in the UCRB is of great ecological concern because hybrids have the capacity to negatively impact vulnerable native species. For example, the hybrid swarm has been expanding into the Upper Conasauga River where the largest population of federally threatened blue shiner (Cyprinella caerulea) naturally occur (Glotzbecker et al., 2016 B).

Hybridization between native and non-native fishes can lead to the demise of the native species in one of two ways. First, if the fitness of F₁ hybrids is greatly reduced compared to parentals, and hybridization occurs frequently, demographic swamping can occur (Todesco et al.,
This happens when the population growth rate of one or both parental species declines, eventually leading to the loss of both species. For example, demographic swamping has been observed in Black bream (*Acanthopagrus butcheri* Munro) and Yellowfin bream (*Acanthopagrus australis*) in Southeastern Australia (Roberts et al., 2009). Hybridization is so common that demographic swamping threatens the integrity of yellowfin bream populations (Roberts et al., 2009). The second pathway that can lead to the loss of native species is through a process known as genetic swamping. This phenomenon typically occurs when hybrid fitness is equal or higher when compared to parental species, and hybrid population growth rates exceed parental replacement rates (Todesco et al., 2016). Consequently, these events could result in one or both parental species becoming genetically replaced by intermediate hybrids (Rosenfield et al., 2004; Walters et al., 2008; Blum et al., 2010; Ward et al., 2012; Glotzbecker et al., 2016 B).

Currently, it is known that the ultimate consequences of hybridization between native and non-native species largely depends on the relative fitness of the resulting hybrids. Generally, hybridization between divergent species is often thought to result in hybrid offspring with lower fitness when compared to parentals (Stelkens et al., 2015). This interpretation is largely based on the classical Dobzhansky-Muller model, which suggests the recombination of alleles from two distinct populations will often result in negative epistatic interactions and incompatibilities (Dobzhansky, 1936; Muller, 1942; Orr, 1997). However, over the past two-decades, the data presented in the literature assessing the long-term fitness consequences of hybridization is conflicting (Stelkens et al., 2015). For example, Echelle and Connor (1989) found that over five years, on a 430 river-kilometer portion of the Pecos River (Texas, USA), populations of native Pecos pupfish (*Cyprinodon pecosensis*) had been completely replaced by a swarm of non-native sheepshead minnow (*Cyprinodon varigatus*) x Pecos pupfish hybrids. Additionally, Muhfeld et
al. (2009) observed that introduced rainbow trout (*Oncorhynchus mykiss*) in Montana, USA, will readily hybridize with native cutthroat trout (*Oncorhynchus larkia lewisi*), resulting in post-F$_1$ hybrids with nearly a fifty-percent reduction in fitness when compared to parentals. However, it was also observed that F$_1$ hybrids have relatively high fitness compared to non-hybrid trout, which could result in the decline of native cutthroat trout populations (Muhfeld et al., 2009).

Invasive red shiner populations provide an excellent opportunity to further study the fitness of hybrids in nature. Previous research conducted by Walters et al., (2008); Blum et al., (2010); Ward et al., (2012); and Glotzbecker et al., (2016 A&B) have largely focused on describing the hybrid system, examining population genetics, monitoring hybrid swarm movement, reconstructing invasion histories, and exploring the mechanisms driving hybridization between invasive red shiner and native blacktail shiner. Previous studies have also compared general morphology of blacktail shiner, red shiner, and hybrids (Walters et al., 2008). However, little is known about the relative fitness of red x blacktail hybrids compared to parentals. This is an important question to address as previous studies found evidence of hybrid shiner driving geographic spread within the UCRB (Blum et al., 2010; Ward et al., 2012; Glotzbecker et al., 2016 B)). Furthermore, analysis of microsatellite data across seven years has recovered post-F$_1$ and backcrossed hybrid genotypes (Walters et al., 2008; Blum et al., 2010; Ward et al., 2012; Glotzbecker et al., 2016 B), suggesting that hybrid shiner may possess competitive levels of fitness. In this study, we evaluated the fitness of red, blacktail, and red x blacktail hybrids by using laboratory-based swimming trials as a proxy of Darwinian fitness. We then developed a suite of regression models to identify likely predictors of maximum swimming velocity for both parentals and hybrids. Lastly, morphological comparisons were conducted
using landmark-based geometric morphometrics to reveal potential relationships between morphology and maximum swimming velocity.

**METHODS**

*Study species*

Red shiner (*C. lutrensis*) are native to the Great Plains, the Central Lowland tributaries of the Mississippi River, and to the Western Coastal Plain drainages of the Rio Grande River Basin (USA) (Glotzbecker et al., 2016 A). Currently, red shiner have been introduced across eleven states outside of their native range in North America (Fuller et al., 1999). Consequently, invasive red shiner have been identified as the second greatest threat to the welfare of native South-Western fishes (Dill and Cordone 1997). Unlike many *Cyprinella* species, red shiner can thrive in areas of low water flow, high turbidity, poor water quality and can aggressively colonize disturbed habitats (Cross and Cavin, 1971; Matthews, 1985, Matthews and Hill, 1977, 1979). Additionally, red shiner are highly fecund, can spawn several times per year and reach sexual maturity within their first year (Gale, 1986; Cross and Collins, 1995; Marsh-Matthews et al., 2002). Introduced red shiner can severely impact native fish populations through competitive habitat displacement (Douglas et al., 1994) and predation on fry (Bestgen et al., 2006; Carpenter and Mueller, 2008). It has also been demonstrated that invasive red shiner have the capacity to hybridize with several native *Cyprinella* species (*C. analostana, C. camura, C. callitaenia, C. spilotpera, C. venusta cercostigma, C. v. stigmatura, C. v. venusta, and C. whipplei*) within non-native ranges, which threatens the genetic integrity of native species (Page and Smith, 1970; Wallace and Ramsey, 1982; Walters et al., 2008; Blum et al., 2010; Marsh-Matthews et al., 2011).
**Invasion history**

Red shiner are one of the most widespread and abundant minnow species in Central North America (Matthews, 1980, 1985). Red shiner were first observed outside of their native range in the Lower Colorado River in the 1950’s and were then introduced to parts of the Eastern United States (Lower Yadkin River and the Upper Coosa River) during the 1970’s (Fuller et al., 1999; Walters et al., 2008). Prior studies suggest that introduced populations in the Eastern and Western United States are most likely the result of multiple independent introduction events (Glotzbecker et al., 2016 A). Similarly, it is thought that the spread of invasive red shiner within drainage basins is primarily from independent introduction events (Glotzbecker et al., 2016 A), and are further driven by secondary introductions via bait bucket or aquarium release (Jennings and Saiki, 1990; Walters et al., 2008; Glotzbecker et al., 2016 A). Invasive red shiner were first observed in the UCRB (Weise Lake, Alabama, USA) in 1974 (Walters et al., 2008), and annual surveys in 1998 first documented hybridization between invasive red shiner and native blacktail shiner in the Upper Coosa River (Burkhead and Huge, 2002; Walters et al., 2008). From these surveys it was determined that the leading edge of the hybrid swarm was moving upstream at a rate of approximately three km per year (Walters et al., 2008). Subsequent sampling in the UCRB revealed that more than one third of sampled individuals exhibited genetic admixture in 2011 (Glotzbecker et al., 2016 B).

**Specimen collection and husbandry**

Field collections of red (n=28), blacktail (n=20), and red x blacktail hybrid shiner (n=20) were conducted during the Fall of 2020 and from April to November of 2021. Collection locations for genetically pure red and blacktail shiner were selected in areas previously established by Glotzbecker et al., (2015). To ensure the parentals used were not backcrossed
hybrids, the parentals collection areas were well out of the range of the hybrid zone. Red shiner were collected from Sandy Creek (33°46'45.1"N 84°29'57.5"W) in Atlanta, Georgia, USA. Blacktail shiner were collected from the Conasauga River (34°57'42.8"N 84°47'22.1"W) in Murray County, Georgia, USA. Red shiner x blacktail shiner hybrids (Figure 1) were collected from three locations along the Coosa River near Rome, Georgia, USA, and Lake Weise in Centre, Alabama, USA (34°10'55.25"N 85°44'5.59"W; 34° 9'56.04"N 85°23'45.89"W; 34°15'7.10"N 85°22'49.16"W).

Live fish were collected using a 3 m x 2 m seine net and temporarily held in a standard 18.9-liter bucket. Collected individuals <38 mm in standard length were released due to difficulties associated with obtaining accurate morphological data. Once sampling was complete, specimens were moved into a 189-liter polyethylene transport tank that was filled with approximately 95-liters of clean water that was conditioned with Stress Coat™ and Ammo Lock™ (API®; Chalfont, PA, USA). Transport tanks were aerated with a 12-volt Fish Saver® (Marine Metal Products; Clearwater, FL, USA), to maintain dissolved oxygen levels. Live specimens were then transported to Georgia College and State University (Milledgeville, GA, USA) and placed into 360-liter glass quarantine tanks (121.9 x 60.9 x 43.1 cm). Specimens were quarantined for a period of two-weeks. Quarantine tanks were furnished with approximately 22.6 kilograms of pea gravel to create a more natural, stress reducing environment. During quarantine, the fishes were fed twice a day with Antibiotic Red and Antibiotic II Orange flake food (Angels Plus; Olean, NY) to prevent bacterial and fungal infections. Post-quarantine, the fishes were then fed Tetra Goldfish flake food twice per day (Tetra, Melle, Germany). Quarantine tanks were cleaned weekly, replacing approximately 50% of the tank volume with clean, filtered tap water, and the gravel substrate was siphoned to remove debris bi-weekly. Local tap water was filtered
through a commercial three-stage sediment and activated carbon filter (FiltersFast; Charlotte, NC, USA). Filtered water was also conditioned with Seachem Prime® (Seachem; Madison, GA, USA) at a rate of 1mL per 38-liters of water. Water quality was tested weekly using a Freshwater Master Test Kit (API®; Chalfont, PA, USA). Water temperature was regulated using central heating and cooling, and was maintained between 23-25°C.

**Fitness and swimming trials**

The fitness of an organism is determined by its reproductive success and reflects how well an organism is adapted to its environment (Rosenberg, 1983). In aquatic habitats, swimming performance is often a characteristic that is related to the survival of many aquatic organisms (Jones et al., 1974; Taylor and McPhail, 1986; Young and Cech, 1993; Stobutzki and Bellwood, 1994; Swanson, 1998). Since most fishes lack weapons to protect themselves from predators, swimming is often used to avoid and survive attacks (Videler, 1993; Reidy et al., 1995; Watkins, 1996). According to Drucker (1996), maximum swimming performance (velocity) can strongly influence the ability of fishes to find food, locate mates, and avoid danger. Since it is often difficult to track individual life histories, many studies have previously used swimming performance in fishes a proxy for Darwinian fitness (Hill and Grossman, 1993; Reidy et al., 2000; Ricón et al., 2007; Johnson et al., 2008).

A custom 23.3-liter recirculating flow chamber was used to measure the swimming performance of parental and hybrid shiner. The main chamber was constructed of 10.1 cm inside-diameter PVC pipe and acrylic plastic. Water velocity was controlled by a ¼ hp Dayton model 4Z248B variable speed magnetic motor (Dayton Electric MFG. CO., Chicago, IL, USA). With the variable speed motor, an amps/water velocity curve was created using a Flow Probe model FP111, digital flow meter (YSI Incorporated, Yellow Springs, OH, USA). 24-hours before
a swimming trial, a 379-liter PVC tank was filled with filtered tap water. The water was then conditioned to with Seachem Prime® and heated to a range of 23-25°C with an Eheim Jager, 300-watt aquarium heater (Eheim, Deizsau, Germany). Conditioned water was then pumped into the flow chamber during swim trials by a Mag-Drive 5 submersible water pump (Danner Mfg., Islandia, N.Y., USA). Both water temperature and dissolved oxygen (DO) was maintained throughout the trials and was measured using a YSI 456 handheld meter (YSI Incorporated, Yellow Springs, OH, USA). After each swimming trial, the flow chamber was drained and refilled with fresh, conditioned water.

Using methodology similar to Brett (1964, 1967) and Farrell (2008), maximum swimming velocity \( U_{\text{max}} \) was measured for twenty-eight red shiner, twenty blacktail shiner, and twenty red x blacktail hybrids. Maximum swimming velocity \( U_{\text{max}} \) was defined as the velocity at which the fish could no longer maintain water position in the flow chamber. Individuals were randomly netted from quarantine tanks and moved into the flow chamber for a twenty-minute acclimation period before a trial. The maximum swimming velocity of each fish was then measured by increasing the water velocity by 0.05 m/s in ten-second intervals, until the fish could no longer maintain swimming position. Each trial was video recorded using an iPhone 11 ProMax (Apple Inc., Cupertino, CA, USA) that was positioned above the flow chamber. After the completion of a trial, individuals were euthanized with Tricane-S at a concentration of 0.001g/mL (Syndel, Ferndale, WA, USA) and preserved in 95% ethanol for morphological analysis. Following morphological analysis, individuals were sexed via dissection.

**Quantitative trait measurement**

Following Boschung and Mayden (2004); Walters et al., (2008); and Ward et al. (2012) a set of phenotypic traits were measured to differentiate between red, blacktail, and red x blacktail,
hybrid shiner. Standard length (SL), maximum body depth (BD), number of lateral line scales, and caudal spot intensity were measured for all specimens. All measurements were taken on the left side of each specimen. Standard length was measured from the tip of the snout to the base of the caudal peduncle using Mitutoyo CD-8” ASX digital calipers (Mitutoyo, Sakado, Takatsu-ku, Kawasaki, Kanagawa, Japan). Maximum body depth (BD) was measured from the anterior insertion of the dorsal fin to the anterior insertion of the anal fin. Established standard length to body depth ratios for each species (red shiner < 4:1; blacktail shiner > 4.75:1; hybrid shiner 4 - 4.75:1) were followed for species identification (Gibbs, 1957; Boschung and Mayden, 2004; Walters et al., 2008, Ward et al., 2012; Hockaday and Geheber, 2020). Caudal spot intensity was scored on a scale of 0-3, zero representing no caudal spot and three representing maximum intensity. Scores of one and two were given to differentiate between feint and intermediate caudal spot intensity. Lateral line scales were also counted using a Luxeo 6Z dissecting microscope (Labomed, Fremont, CA). As established in Walters et al. (2008), Ward et al. (2012), and Hockaday and Geheber (2020), hybrid shiner were positively identified by the presence of intermediate or incongruent morphological traits when compared to parental species.

**Landmark-based geometric morphometrics**

Following swim trials, preserved specimens were individually photographed for morphological analysis. Using a Nikon D3500 camera, (Nikon; Tokyo, Japan) all specimens were photographed on the left side. Using methods from Akers and Gehebers (2020), a scale bar was included in each photograph so size differences could be accounted for in the analysis of body shape. Homologous landmark points were then selected following the research by Hass et al. (2015). In R, the software package “SteroMorph” (Olsen & Haber, 2021), was used to digitize ten homologous landmark points for each specimen. The selected points have been identified to
represent a comprehensive convergence of a fish’s lateral form, and they consist of anatomical locations on a single plane that are repeatable (Zelditch et al., 2012). All landmark points were placed by one individual for consistency. To place the data points onto a common plane, a Generalized Procrustes Alignment (GPA) was used to rotate, translate, and scale the data to a best fit model. This procedure was completed using the “geomorph” package in R (Adams et al., 2018; R Development Core Team, 2022). A Principal Component Analysis (PCA) was then used to reduce the ten morphological landmarks into linearly uncorrelated variables that would explain the greatest amount of variation among the three species. The PCA was ultimately used to illustrate the morphological variance between red shiner, blacktail shiner, and hybrids.

**Identifying predictors of swimming performance**

All data analyses were performed using the R programming language (R Development Core Team 2022). Maximum swimming velocity is influenced by many biological characteristics that may be indicative of fitness differentials between parental and hybrids, including sex, body morphometrics, body length, and species assignment (Jones et al., 1974; Taylor and McPhail, 1986; Young and Cech, 1993; Stobutzki and Bellwood, 1994; Swanson, 1998). We fit a suite of linear effects models to identify predictors of maximum swimming velocity. A total of fifteen models were evaluated, which modeled maximum swimming velocity as a function of one or more factors, including: sex, body depth, standard length, and species (Appendix A). Using Akaike Information criterion (AIC) and Bayesian Information Criterion (BIC) analyses, regression models with interaction effects were compared to identify “best fit” models to explain our data. Models were also tested by looking at the R² to determine the absolute model fit. Best-fit models were selected using the smallest AIC values and the largest R² values and models with AIC/BIC values two units lower than other models were considered significant (Johnson et al.,
Lastly, to account for size differences contributing to differences in swimming performance, we performed a linear regression analysis of swimming velocity against standard length for each individual. An ANOVA was then run using the residuals values obtained from the correlation analysis, with species assignment as the predictor variable.

RESULTS

Species differences in morphology

We observed distinct morphological differences between red, blacktail, and hybrid shiner (Table 1). On average, blacktail shiner were the largest of the three species (SL \( \bar{x} \) 63.18mm), red shiner were the smallest (SL \( \bar{x} \) 46.35mm), and hybrid shiner were intermediate of the two parental groups (SL \( \bar{x} \) 53.46mm), (Table 1). A comparison of standard length to body depth ratios (SL/BD) revealed a similar trend with blacktail shiner having the largest ratio (SL/BD \( \bar{x} \) 4.54), red shiner the smallest ratio (SL/BD \( \bar{x} \) 3.30), and hybrid shiner were once again intermediate of the two parental species (SL/BD \( \bar{x} \) 4.08), (Table 1). However, an ANOVA comparing standard length to body depth between the three populations did not indicate significant differences between populations \( (P = 0.22) \). Lateral line scale counts were significantly different among the three species \( (P = 0.011) \), on average blacktail shiner had 40.3 scales, red shiner 34.19 scales, and hybrids an intermediate count of 36.1 scales (Table 1). Overall, our measurements are consistent with previous morphological work conducted on the three populations (Boschung and Mayden, 2004; Walters et al., 2008; Ward et al., 2012).

Dissection and sexing of the specimens revealed that relatively even sex ratios were maintained among the three species groups. For both blacktail and red shiner, we observed an even 1:1 ratio of males to females, while the sexing of hybrids resulted in a ratio of eleven males
to nine females. Standard length did not significantly differ between blacktail shiner males and females (\(\bar{x}_{0.33\text{mm}} = 63.04\text{mm} \); \(P > 0.93\), (Table 1). However, standard length for red shiner males and females differed significantly based on t-tests (\(\bar{x}_{47.96\text{mm}} = 64.75\text{mm} \); \(P = 0.04\), (Table 1). Hybrid males were typically larger than females (\(\bar{x}_{55.75\text{mm}} = 65.66\text{mm} \)), (Table 1), however the difference was not significant (\(P > 0.05\)). Body depth was not significantly different between blacktail shiner females and males (\(\bar{x}_{14.01\text{mm}} = 13.67\text{mm} \); \(P > 0.05\), (Table 1). Similarly, red shiner males were significantly deeper bodied than females (\(\bar{x}_{15.53\text{mm}} = 12.55\text{mm} \); \(P < 0.001\)), and hybrid males did not have significantly deeper bodies than hybrids females (\(\bar{x}_{13.87\text{mm}} = 12.15\text{mm} \); \(P = 0.11\), (Table 1). Finally, lateral line scale counts were not significantly different between the sexes for blacktail, red, and hybrid shiner (\(\bar{x}_{39.9} = 40.7\), \(\bar{x}_{34} = 34.36\), \(\bar{x}_{36.82} = 35.22\), (Table 1).

**Interspecific analysis of landmark-based geometric morphometrics**

Here we present all outcomes from the geometric morphometric analysis based on the entire data set. There was no exclusion of data, however, we included eight more red shiner than blacktails and hybrids to maintain an approximately equal sex ratio. As illustrated in Figure 4, the morphology of the three species is distinct. Red shiner are deeper bodied than blacktails and hybrids, and blacktail shiner are more streamlined compared to the other two species (Table 1). Hybrids exhibit an intermediate body shape compared to the two parental species, having a slightly deeper body compared to blacktail shiner but not as deep as red shiner. Principal component analysis (PCA) resulted in five principal components (PC) that explained 78.36% of shape variation within the three species (PC1 = 40.503%, PC2 = 16.544%, PC3 = 9.238%, PC4 = 6.993%, PC5 = 5.079%). Additional PC values explained <5% of the variation and were not interpreted. PC1 is correlated with the posterior edge of the operculum, PC2 the tip of the snout,
PC3 the anterior pelvic fin insertion, PC4 the nape, and PC5 the anterior dorsal fin insertion. A one-way ANOVA revealed a significant difference between the location of the posterior edge of the operculum (PC1) for the three species ($P < 0.001$), (Figure 5).

**Species differences in maximum swimming velocity**

Evaluation of maximum swimming velocity ($U_{\text{max}}$) revealed significant differences among the three species groups ($P = 0.021$), (Figure 6). Blacktail shiner exhibited the highest $U_{\text{max}}$ ($\bar{x}$0.90 m/s), followed by red shiner x blacktail shiner hybrids, ($\bar{x}$0.89 m/s), and red shiner were the slowest with an average of 0.78 m/s (Table 2). When considering sex, red shiner x blacktail hybrid males ($U_{\text{max}} \bar{x}$0.93 m/s) had higher maximum swimming velocities than both blacktail and red shiner males, however it is insignificant ($\bar{x}$0.86 m/s; 0.88 m/s $P = 0.33$), (Table 2). Female blacktail shiner exhibited the highest $U_{\text{max}}$ of all the species examined ($U_{\text{max}} \bar{x}$0.94 m/s), and female red shiner were the slowest in our trials, with an average velocity of 0.69 m/s, (Table 2). Red x blacktail shiner hybrid females were intermediate in $U_{\text{max}}$ when compared to parental groups ($U_{\text{max}} \bar{x}$0.85 m/s), (Table 2). Female red shiner $U_{\text{max}}$ was significantly slower when compared to red shiner males ($P =0.004$), (Figure 7).

For both AIC and BIC analyses, $U_{\text{max}}$ was best predicted by (body depth) x (species) (model 11, Appendix A, AIC = -66.02; BIC = -57.14, $R^2$ = 0.24). Two additional models predicted $U_{\text{max}}$ as a function of (body depth) x (sex) x (species) (model 2, Appendix A, AIC = 64.17, $R^2$ = 0.23) and (body depth) x (standard length) x (species) (model 4, Appendix A, AIC = 64.09, $R^2$ = 0.23). Other models were significantly weaker compared to model 11 ($\Delta$AIC >2) and were not considered for further analysis. Notably, sex was only a significant predictor of $U_{\text{max}}$ for red shiner ($p = 0.004$), (Figure 7). In agreement with our top AIC model ANOVA analysis
revealed that standard length was not a significant predictor of $U_{\text{max}}$ for all species groups ($P = 0.065$).

**DISCUSSION**

Overall, our results suggest that red x blacktail shiner hybrids demonstrate intermediate swimming performance when compared to parental species. Hybrid shiner also expressed intermediate morphological characteristics when compared to both parental shiner. This trend was observed in standard length, SL/BD ratios, lateral line scale counts, and landmark based geometric morphometric analyses (Figure 4). Similarly, our swimming trials imply that there is a significant relationship between morphology and maximum swimming velocity among the three species. On average, hybrid shiner exhibited maximum swimming velocities that were intermediate of parental red and blacktail shiner. Our swimming trials also indicate that hybrid shiner do not significantly differ from parental blacktail shiner in maximum swimming velocity. Notably, hybrid males were able to achieve higher maximum swimming velocities when compared to both red and blacktail shiner males. When examining maximum swimming velocity among female groups, hybrid female shiner achieved a higher average velocity than did female red shiner, but were slower when compared to female blacktail shiner. Our swimming trials also revealed that female blacktail shiner achieved the highest maximum swimming velocity and female red shiner the lowest. In our analyses, we did not find a significant correlation between standard length and maximum swimming velocity. Our models suggest that swimming velocity was best predicted by factors such as species and body depth. Our results suggests that hybridization events between native blacktail shiner and invasive red shiner can produce hybrids that demonstrate equal or higher fitness when compared to parental species. Consequently, it is
likely that red x blacktail shiner hybrids pose a greater threat to native *Cyprinella* species than parental red shiner.

*Factors explaining differences in maximum swimming velocity*

The genetic and physiological elements that influence the maximum swimming speed of freshwater teleosts remains poorly understood. In a recent meta-analysis, researchers examined 204 studies assessing the maximum swimming speed of thirty-five species of freshwater fishes native to the Iberian Peninsula (Cano-Barbacil et al. 2020). The results suggest that body length, taxonomic family, trial speed interval and species were the largest predictors of maximum swimming speed. In contrast to this study, our results examining cyprinids endemic to North America did not conclude that body length was a significant predictor of maximum swimming velocity. However, the differences in maximum swimming velocities that we observed were better predicted by factors such as species and body depth. Our results consistent with previous studies that have highlighted the importance of body depth in swimming performance. Specifically, morphological features that maximize thrust and stability are typically produced by a deep body and a low aspect-ratio caudal fin (Webb, 1984; Domenici et al., 2008). Notably, sex was only a significant predictor of maximum swimming velocity for red shiner. A possible explanation for this difference is that many of the female red shiner were gravid at the time of testing. Consequently, gravid females are physiologically different than non-gravid females and a significant proportion of their energy is being diverted towards oogenesis. Another potential explanation is that red shiner are considerably more sexually dimorphic compared to blacktail shiner. Male red shiner are deeper bodied when compared to female red shiner and this difference could result in males being able to generate more thrust and having greater swimming stability.
Effects of competitive fitness among hybrid *Cyprinella* in non-native ranges

It is known that the swimming performance of most fishes is strongly correlated with their ability to acquire food, find mates, and survive (Drucker, 1996; Plaut, 2001). Accordingly, it is suggested that swimming ability is a critical adaptive trait that influences fitness (Reidy et al., 2000; Plaut, 2001). In this capacity, our data suggests that the production of red x blacktail shiner hybrids with equal or greater fitness, compared to parental species, may result in higher invasion rates, more rapid spread, and an overall decline in native *Cyprinella* diversity.

Consistent with similar studies, we observed that red x blacktail shiner hybrids exhibit similar fitness when compared to parental species. For example, Rosenfield et al., (2004) found that hybrids produced by endemic Pecos pupfish (*Cyprinodon pecosensis*) and introduced sheepshead minnows (*Cyprinodon variegatus*) exhibited intermediate swimming performance when compared to parentals. Similarly, Blum et al., (2010) showed that *C. lutrensis* x *C. venusta* hybrids have equal or higher postzygotic fitness than offspring from parental species under laboratory conditions. Our findings are also supported by a meta-analysis of research, suggesting that hybrid phenotypes often confer higher fecundity and equal survival when compared to parental species (Hovick and Whitney, 2014; Glotzbecker et al., 2016 B). Similarly, Johnson et al., (2014) demonstrated that naturally occurring swordtail hybrids (*Xiphophorus*) produce offspring that are intermediate in fast start swimming when compared to parentals (*Xiphophorus malinche* and *X. birchmanni*). However, in this case, the hybrids also displayed the greatest swimming endurance when compared to parental groups. Unlike our results, it was also suggested that that the morphology of *X. birchmanni* and hybrid male groups did not play a significant role in their swimming performance (Johnson et al., 20014).
Examining the literature, it is also important to mention that many of the results from prior studies assessing the swimming performance of hybrid and parental fishes are somewhat inconclusive, when looking for evidence of hybrid vigor. For example, Rouleau et al., (2010) showed that hybrid brook trout (*Salvelinus frontinalis*) had lower critical swimming speeds across a range of water temperatures, when compared to parentals. These observations are also reflected in the work of Mee et al. (2011), which suggests that asexually reproducing hybrid dace (*Phoxinus*) had significantly lower swimming performance compared to the sexually reproducing parental species, the fine scale dace (*Phoxinus neogaeus*).

**Expanding hybrid swarms and native Cyprinella conservation**

To our knowledge, this study is one of the few examples illustrating elevated hybrid fitness among freshwater stream fishes. With the potential of *C. lutrensis x C. venusta* hybrids having equal or greater fitness compared to parental species, there is a strong likelihood that future hybridization events will accelerate the spread of invasive red shiner and exacerbate the loss of native *Cyprinella* within the UCRB. Based on prior work by Glotzbecker et al., (2015), it has been established that elevated turbidity can disrupt prezygotic isolating barriers between red and blacktail shiner, resulting in the production of large *C. lutrensis x C. venusta* hybrid swarms. It has also been documented that the *C. lutrensis x C. venusta* hybrid swarms can move rapidly within large river systems and spread hundreds of kilometers in less than a decade (Walters et al., 2008; Ward et al., 2012; Glotzbecker et al., 2016B). Earlier studies (Walters et al., 2008; Blum et al., 2010; Ward et al., 2012; Glotzbecker et al., 2017) have strongly believed that the spread of invasive red shiner was largely due to genetic introgression and the formation of hybrid swarms. The results of this study validate prior theories, as elevated hybrid fitness will result in increased competition with native *Cyprinella*, possibly resulting in localized extinction events. In time, the
spread of invasive red shiner via hybrid swarms could also compromise small populations of threatened or endangered species, such as the federally threatened blue shiner (*C. caerulea*) (Glotzbecker et al., 2016B). The Conasauga River contains the largest remaining population of blue shiner, and the presence of non-native competitors such as red shiner and *C. lutrensis x C. venusta* hybrids could pose a major conservation threat. Continued monitoring of the UCRB should remain a top priority to protect native *Cyprinella* species. Additionally, in localities with elevated turbidity and high *C. lutrensis x C. venusta* hybrid capture rates, efforts to improve water quality should be implemented at local and state levels.
ACKNOWLEDGEMENTS

This project was a lot of work and required the help of many extra hands. I would like to thank Dr. Glotzbecker for his help and guidance throughout the entire process. I would also like to thank Dr. Adams for all the help he gave me with RStudio. I honestly do not know how I would have been able to learn RStudio without all his help. I want to thank Dr. Weese for his encouragement and support throughout the process. I also want to thank Dr. Milnes for always questioning my methods and making sure that we were accounting for everything. These committee members helped me grow and I really appreciate all their help and patience. I also want to thank Dr. Grossman who let me borrow his flow chamber. I also want to give a huge thank you to everyone that went on countless fish collecting trips. I would not have been able to finish without all your help. I would also like to thank my family and friends for all their support throughout this entire process.
REFERENCES


Table 1: Averages of general morphology measurements for red, blacktail, and hybrid shiner. Standard Length (SL), Body Depth (BD), Lateral Line Scale Count (LLSC).

<table>
<thead>
<tr>
<th>Species</th>
<th>SL (mm)</th>
<th>Male SL (mm)</th>
<th>Female SL (mm)</th>
<th>BD (mm)</th>
<th>Male BD (mm)</th>
<th>Female BD (mm)</th>
<th>LLSC</th>
<th>Male LLSC</th>
<th>Female LLSC</th>
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<tr>
<td>Cyprinella venusta</td>
<td>63.18 (±6.90)</td>
<td>63.33 (±8.77)</td>
<td>63.04 (±4.87)</td>
<td>13.89 (±1.64)</td>
<td>13.67 (±2.07)</td>
<td>14.01 (±1.14)</td>
<td>40.3 (±1.89)</td>
<td>39.9 (±2.02)</td>
<td>40.7 (±1.77)</td>
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<tr>
<td>Cyprinella lutrensis</td>
<td>46.35 (±4.25)</td>
<td>47.95 (±3.70)</td>
<td>44.75 (±4.29)</td>
<td>14.04 (±1.85)</td>
<td>15.53 (±1.32)</td>
<td>12.55 (±0.78)</td>
<td>34.18 (±1.12)</td>
<td>34 (±1.18)</td>
<td>34.36 (±1.08)</td>
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<tr>
<td>C. venusta x C. lutrensis</td>
<td>53.46 (±7.57)</td>
<td>55.75 (±8.28)</td>
<td>50.66 (±5.88)</td>
<td>13.1 (±2.39)</td>
<td>13.87 (±2.67)</td>
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<td>36.1 (±2.07)</td>
<td>36.82 (±1.99)</td>
<td>35.22 (±1.92)</td>
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Table 2: Average maximum swimming velocities for red, blacktail, and hybrid shiner.

<table>
<thead>
<tr>
<th>Species</th>
<th>Velocity (m/s)</th>
<th>Male velocity (m/s)</th>
<th>Female velocity (m/s)</th>
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<td>Cyprinella venusta</td>
<td>0.90 (±0.13)</td>
<td>0.86 (±0.16)</td>
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<td>Cyprinella lutrensis</td>
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<tr>
<td>C. venusta x C. lutrensis</td>
<td>0.89 (±0.14)</td>
<td>0.93 (±0.12)</td>
<td>0.85 (±0.15)</td>
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Figure 1: Photographs of: (A) Red Shiner, (B) Red Shiner x Blacktail Shiner Hybrid, (C) Blacktail Shiner.

Figure 2: Diagram of the recirculating flow chamber used for swimming trials. Motor (A), shaft (B), impeller (C). Flow chamber diagram, arrows indicate the direction of water flow.
Figure 3: The 10 landmark (LM) locations identified in *Cyprinella venusta* and *Cyprinella lutrensis*: (1) tip of the snout, (2) center of the eye, (3) nape, (4) anterior dorsal fin insertion, (5-6) dorsal and ventral caudal fin insertion, (7) anterior anal fin insertion, (8) anterior pelvic fin insertion, (9) base of the isthmus, and (10) the posterior edge of the operculum.
Figure 4: Generalized Procrustean Analysis (GPA) for body shape of the 3 species. Black points represent an average for individuals and grey points represent individuals. Each of the 10 landmarks and where the general placement was for each of the different populations.
Figure 5: Relationship between PC1 and PC2 for red, blacktail, and hybrid shiner. Red circles = *Cyprinella lutrensis*, black circles = *Cyprinella venusta*, and gray circles = *Cyprinella lutrensis* x *Cyprinella venusta* hybrids.
Figure 6. Jitter plot comparing the maximum swimming velocity for red, blacktail, and hybrid shiner. Black dots represent female while gray dots represent males.
Figure 7. Comparison of maximum swimming velocities by sex for *Cyprinella lutrensis*. 
Appendix A. AIC, BIC, and $R^2$ for absolute model fit were used to identify predictors of maximum swimming velocity for red, blacktail, and hybrid shiner. AIC and BIC scores marked with an asterisk (*) are significant.

<table>
<thead>
<tr>
<th>Model</th>
<th>Predictors</th>
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<th>BIC Score</th>
<th>$R^2$</th>
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<td>0.23*</td>
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