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## Article

# The roles of inter- and intra-sexual selection in behavioral isolation between native and invasive pupfishes

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## Abstract

Male–male competition and female mate choice may both play important roles in driving and maintaining reproductive isolation between species. When previously allopatric species come into secondary contact with each other due to introductions, they provide an opportunity to evaluate the identity and strength of reproductive isolating mechanisms. If reproductive isolation is not maintained, hybridization may occur. We examined how reproductive isolating mechanisms mediate hybridization between endemic populations of the Red River pupfish *Cyprinodon rubrofluviatilis* and the recently introduced sheepshead minnow *C. variegatus*. In lab-based dominance trials, males of both species won the same number of competitions. However, male *C. rubrofluviatilis* that won competitions were more aggressive than *C. variegatus* winners, and more aggression was needed to win against competitor *C. variegatus* than allopatric *C. rubrofluviatilis*. Duration of fights also differed based on the relatedness of the competitor. In dichotomous mate choice trials, there were no conspecific or heterospecific preferences expressed by females of either species. Our findings that male–male aggression differs between closely and distantly related groups, but female choice does not suggest that male–male competition may be the more likely mechanism to impede gene flow in this system.

**Key words:** *Cyprinodon*, female mate choice, invasive species, male–male competition, reproductive isolation, sexual selection.

Formerly allopatric species are increasingly coming into secondary contact due to human introduction (Rhymer and Simberloff 1996) allowing us to test how mechanisms of reproductive isolation have evolved and make inferences to the speciation process (Sax et al. 2007; Ward and Blum 2012; Lackey and Boughman 2013b; Heathcote et al. 2016). When populations are geographically isolated, independent evolutionary pressures can reduce reproductive compatibility between populations (Wang 2013), but reproductive isolation may not be maintained if populations come into secondary contact (Gilman and Behm 2011). The mechanisms mediating gene flow upon secondary contact are often not understood except in stable hybrid zones where other processes, such as reinforcement have occurred (Harrison 1993; Dowling and Secor 1997; Servedio and Noor 2003; Seehausen 2004; Mallet 2005). Reproductive isolating

mechanisms may either promote or impede the process of hybridization and therefore, play an important role in mediating the effects of invasive species on native relatives.

Reproductive isolation can be driven by one or multiple isolating mechanisms (reviewed in Coyne and Orr 2004; Ramsey et al. 2003; Blum et al. 2010; Berdan and Fuller 2012; Gregorio et al. 2012). When species are sexually isolated, both intra-sexual selection (male–male competition) and inter-sexual selection (female mate choice; Darwin 1871) may decrease gene flow between populations (Boughman 2001; Servedio 2004; Qvarnström et al. 2012). In systems where male–male competition occurs, males actively compete for access to females, or the resources that are necessary to attract females. For example, if certain habitat types are required for females to deposit eggs, males will compete for space in that habitat,

and defend territories around those resources (reviewed in Andersson 1994; Wong and Candolin 2005). Male competition may impede gene flow, contributing to reproductive isolation. In some systems, this occurs when aggression is biased toward competitors that are phenotypically similar and in other systems divergence is promoted when aggression is biased toward competitors that have more phenotypic differences (Rosenfield and Kodric-Brown 2003; Seehausen and Schluter 2004; Dijkstra et al. 2005; Lackey and Boughman 2013a; Tinghitella et al. 2015). Alternatively, when species have similar breeding habitat and behavior, male competition between species can promote gene flow, leading to introgression between species when aggression facilitates interspecific breeding (Rosenfield and Kodric-Brown 2003). In systems where female mate choice occurs, females choose high-quality mates that provide direct or indirect benefits to them or their offspring (reviewed in Andersson 1994). Traits that determine a high-quality male can differ between species, leading to assortative mating (Lande 1981; West-Eberhard 1983; Boughman 2001; Panhuis et al. 2001; Williams and Mendelson 2011; Williams et al. 2013). Conversely, when sexually selected traits are similar between species, mating between heterospecifics may occur. Further, if an heterospecific has traits that are preferred over those of conspecifics, there is little or no maintenance of reproductive isolation and hybridization will occur (Kodric-Brown and Rosenfield 2004; Abbott et al. 2013). The processes of sexual selection are not mutually exclusive and understanding how multiple selective pressures act on traits is critical to knowing the full extent of the evolution of these traits within a species (Fuller 2003; Reichard et al. 2005; Hunt et al. 2009), as well as how they influence interactions between species and contribute to sexual isolation.

Introductions of the sheepshead minnow *Cyprinodon variegatus* into the ranges of multiple closely related species have essentially replicated recent secondary contact events, providing opportunities to test behavioral mechanisms that contribute to reproductive isolation across the group. The most well-studied case is the invasion of *C. variegatus* in the Pecos River where the Pecos pupfish, *Cyprinodon pecosensis*, was once the most abundant fish species (Echelle and Connor 1989). In the span of five years (1980–84), hybrids of *C. pecosensis* and *C. variegatus* were found in over half of the native geographical range of *C. pecosensis* (Echelle and Connor 1989; Wilde and Echelle 1992). Hybridization with invasive *C. variegatus* was due to a lack of prezygotic isolating mechanisms between the two species. Female *C. pecosensis* preferred heterospecific males in visual preference tests (Kodric-Brown and Rosenfield 2004). During male–male competition trials, male *C. variegatus* were more aggressive than male *C. pecosensis* (Rosenfield and Kodric-Brown 2003). Also, male hybrids showed more aggressive behaviors than males of either species, contributing to the rapid replacement of *C. pecosensis* with hybrids (Rosenfield and Kodric-Brown 2003).

The introduction and introgression of *C. variegatus* has not been limited to the Pecos River system and *C. pecosensis*. *Cyprinodon variegatus* introductions have been documented in populations of the Comanche Spring pupfish *Cyprinodon elegans* (Echelle and Echelle 1994), and the Leon Springs pupfish *Cyprinodon bovinus* (Echelle and Echelle 1997), with varying degrees of gene flow between species. Minimal hybridization and introgression occurred between *C. variegatus* and *C. elegans*. A small hybrid zone became established at the edge of each species range but male hybrids had low fertility (Tech 2006a), lacked gonadal development (Stevenson and Buchanan 1973) and there were increased mortality rates for backcrosses (Tech 2006a). In addition, there may be prezygotic

isolation, with females expressing visual preferences for conspecifics over heterospecifics in both species (Tech 2006b). Although the mechanisms promoting introgression between *C. variegatus* and *C. bovinus* remain unstudied, the entire wild population of *C. bovinus* was introgressed with *C. variegatus* (Echelle and Echelle 1997). Hybridization between these species prompted multiple successful eradication efforts starting in 1976 (Hubbs 1980), but *C. variegatus* introgression had lasting effects on the wild population of *C. bovinus*, comprising 6–15% of the genetic makeup even after eradication (Echelle and Echelle 1997).

Recently, *C. variegatus* has been introduced into the Brazos River (G. Wilde, unpublished data), which is home to the native Red River pupfish *Cyprinodon rubrofluviatilis*. *Cyprinodon rubrofluviatilis* occupies niches throughout its range that are very similar to other *Cyprinodon* species, and therefore the introduction of *C. variegatus* may pose a conservation threat to *C. rubrofluviatilis* if reproductive isolation is not maintained upon secondary contact. There are two populations of *C. rubrofluviatilis* that are geographically isolated with one occurring in the Brazos River, and the other found in the Wichita and Red Rivers. The Red/Wichita River and Brazos River populations of *C. rubrofluviatilis* are genetically distinct and these two forms do not form a monophyletic clade (Echelle and Echelle 1992; Ashbaugh et al. 1994; Echelle et al. 2005; Martin and Wainwright 2011), however they have not been formally recognized as different species. The Brazos River form is more closely related to other species in the southwest (*C. bovinus*, *C. elegans*, *C. pecosensis*) than it is to the Red River form of *C. rubrofluviatilis* (Martin and Wainwright 2011). mtDNA analysis estimates divergence time between *C. variegatus* and the rest of the southwestern *Cyprinodon* spp. to be approximately 4.6 million years (Echelle et al. 2005). Similar to the variation in reproductive isolation across *Cyprinodon* species, populations of *C. rubrofluviatilis* may differ in the identity and strength of behavioral isolating mechanisms with a common heterospecific *C. variegatus*.

We examined the reproductive isolating mechanisms which may mediate hybridization between species that have recently come into secondary contact due to human activity. By evaluating male–male competition and female mate preferences, our objective was to determine if and how each process contributes to sexual isolation. We test both mechanisms of sexual selection between genetically distinct allopatric populations within a species, and between species to further determine the roles of inter- or intra- sexual selection at different levels of divergence.

## Materials and Methods

### Study organisms

*Cyprinodon rubrofluviatilis* and *C. variegatus* are both small, deep-bodied fishes, as is typical of the genus *Cyprinodon*. Pupfishes predominantly inhabit benthic habitats in relatively small bodies of water in the southwestern United States and Central America (Echelle et al. 1972; Echelle et al. 2005). *Cyprinodon rubrofluviatilis* differs from *C. variegatus* in shape, scalation, and coloration (Page and Burr 2011) but both species prefer similar habitat in their native systems that consists of sandy areas with minimal vegetation (Echelle 1973; Hubbs et al. 1991). Both species have a promiscuous breeding system in which males compete to establish spawning territories where they court females (Echelle 1973; Itzkowitz 1977). For *C. rubrofluviatilis*, breeding occurs throughout most of the year, but spawning is more intense during the spring and summer months (Echelle et al. 1972; Lee et al. 2015). Females of other *Cyprinodon*

species are known to prefer larger males, those that have more coloration, and prefer certain territory qualities (Kodric-Brown 1983; Draud 1996; Ludlow et al. 2001). In all *Cyprinodon* species, when females enter the breeding grounds, territorial males display herding and courting behaviors (described by Echelle 1970), whereas females evaluate males and their territories. Females receptive to mating swim to the substrate of the territory, where spawning occurs. Females deposit one egg at a time, but often deposit multiple eggs in a row by spawning with the same male repeatedly. While the egg(s) are laid, the male releases sperm then continues defending the territory. Neighboring males may be attracted by the courting and mating behaviors and try to disrupt the process (Echelle 1970). Due to disruptions by other males, the courtship of the female may not be continuous, but instead be interspersed with quick chases to intruding males. This can be costly to the territorial male, because the female may leave his territory while he is engaged in long aggressive bouts with other males. Similar territorial and mating behaviors are also seen in *C. variegatus* (Itzkowitz 1977; Itzkowitz 1978; Itzkowitz 1981) and other *Cyprinodon* species (Kodric-Brown 1977; Kodric-Brown 1981; Gumm 2012).

### Collection, maintenance, and experimental setup

*Cyprinodon rubrofluvialtilis* and *C. variegatus* in breeding condition were collected 18–19 June 2014, 6–7 September 2014, and 2 April 2015 from rivers in Texas, USA. *Cyprinodon rubrofluvialtilis* were collected from the North Wichita River, a tributary of the Red River, and the Salt Fork of the Brazos River. These populations did not show evidence of hybridization using morphological or genetic methods (Ayers and Gumm, unpublished data). *Cyprinodon variegatus* were collected from the Brazos River directly downstream of Possum Kingdom Reservoir. Fishes were caught using seine nets, and were separated in coolers by species and sex for transportation to Stephen F. Austin State University (SFASU). Fishes were kept in coolers for less than 48 h and water quality (temp, pH, and ammonia) was monitored periodically during transportation. At SFASU, fishes were housed in a dedicated animal facility, where they were maintained at a constant 26.5 °C on a 12L: 12D light cycle. Holding aquaria and experiment trials were illuminated with Coralife™ T5 dual light fixtures (one Colormax 28-watt bulb, one 6700K 28-watt bulb). Fishes were kept in 37-L holding aquaria, separated by species and sex. Each aquarium did not exceed 15 individuals and all aquaria had cardboard dividers between them, limiting visual exposure to other species and sexes before testing.

All experimental studies had a similar setup. A single light fixture was placed approximately 6 inches above the test aquarium. To ensure no distractions of the experimental fish, black construction paper covered the back and sides of the aquarium and two black curtains lined the path from the observer to the aquarium. Trials were recorded with a video camera mounted on a tripod directly in front of the observer. After being a focal individual in an experiment, fishes were placed into separate post-experiment holding aquaria to then be used as stimulus individuals. Stimulus fishes were never used more than once a day, and never paired with the same fish twice for one treatment group.

### Male–male competition trials

Dominance studies were conducted to compare male aggressive behaviors during fights for breeding sites and identify if males of one type win more fights. All trials were conducted in a 37-L aquarium with gravel substrate and a spawning mop in the center, to

encourage territorial behavior. A 9.5-L aquarium was placed along the back of the focal tank with a female *C. rubrofluvialtilis* to further incite territorial behavior over the breeding site. The three treatments for dominance trials were: *C. variegatus* versus Wichita River *C. rubrofluvialtilis* ( $n=14$ ), *C. variegatus* versus Brazos River *C. rubrofluvialtilis* ( $n=15$ ), and Wichita River *C. rubrofluvialtilis* versus Brazos River *C. rubrofluvialtilis* ( $n=10$ ).

For each trial, the two males were placed in the focal tank at the same time. After a 5-min acclimation, the trial began either (1) when five aggressive behaviors occurred in 30 s or (2) after 15 min. This allowed the trial to start when fighting began, as opposed to a set time in most cases. During the trial, all behaviors were recorded as events in Jwatcher (version 1.0), where aggressive behaviors that occurred were chases, bites, and lateral displays. Chases were defined as one male swimming toward the other male with no physical contact observed. A bite was similar to a chase, but included physical contact. Displays were recorded when both males raised their dorsal fins and curved their head and tails in toward each other accompanied by circling behavior. Ultimately, the trial ended when one male chased the other male 10 times without being chased or bitten back, showing clear evidence he was the dominant male in the trial. Determining a winner in this way was done to prevent injury or death to subordinate males by prohibiting prolonged interactions with high levels of aggression. If this threshold of aggression did not occur in a trial, the winner was determined as the male that was clearly defending the spawning territory at the end of 1 h. In one trial, a winner could not be determined by either of these methods, and that trial was removed from the analysis.

For each treatment, a chi-squared test with a null 50: 50 expected ratio was used to evaluate differences in the population identity of winners. A One-Way ANOVA, followed by a Tukey HSD post hoc test was used to test for differences in the lengths of trials between treatments. All aggressive behaviors were standardized per minute due to differences in trial lengths. ANOVAs with a Tukey HSD post hoc test were used to examine differences in aggressive behavior between treatments with factors being the type of male (Brazos River *C. rubrofluvialtilis*, Wichita River *C. rubrofluvialtilis*, or *C. variegatus*) and treatment (competitor identity), and the interaction between the two factors. Males in fights were not always the same size, therefore a paired *t*-test was performed to test if winning males were significantly larger. All tests use  $\alpha=0.05$  as statistical significance.

### Female mate choice trials

Female visual preference experiments used a dichotomous choice setup, consisting of a focal aquarium (37-L) placed lengthwise, with two smaller aquaria (9.5-L) on either side. Interaction zones were marked vertically on front and back of the focal tank 5 cm from the borders adjacent to the two side aquaria. Single males from both groups in the treatment were randomly placed in the small, outer aquaria. Males of relatively equal size (*C. rubrofluvialtilis* Brazos =  $35.41 \pm 1.48$  mm; *C. rubrofluvialtilis* Wichita =  $41.43 \pm 1.38$  mm; *C. variegatus* =  $34.11 \pm 1.94$  mm) were paired as stimuli to account for any behavioral or morphological differences related to size. Males from the Wichita River population of *C. rubrofluvialtilis* were larger on average, but the larger male in a specific trial was never more than 25% larger than the smaller male. There were significant differences in male size in trials, but females did not spend significantly more time with the larger male, regardless of species or population identity (Wilcoxon Signed Rank Test, *C. rubrofluvialtilis* Brazos  $P=0.60$ ; *C. rubrofluvialtilis* Wichita  $P=0.35$ ; *C. variegatus*

$P=0.40$ ). Based on pretrial observations there was no indication that stimulus males reacted to each other or had the ability to gain familiarity with each other due to the distance between aquaria. Therefore, stimulus males were used more than once, but never in the same day nor the same two fish for multiple trials in one treatment, thus alleviating possible pseudoreplication in a treatment. In total, there were four treatments; female Wichita River *C. rubrofluvialis* tested for preference between a conspecific male from her own population (Wichita) and a conspecific from an allopatric population (Brazos,  $n=15$ ), female Wichita River *C. rubrofluvialis* tested for preference between a conspecific male and heterospecific male *C. variegatus* ( $n=15$ ), female Brazos River *C. rubrofluvialis* tested for preference between a conspecific male and heterospecific male *C. variegatus* ( $n=15$ ), and female *C. variegatus* tested for preference between a conspecific male and a male Brazos River *C. rubrofluvialis* ( $n=16$ ).

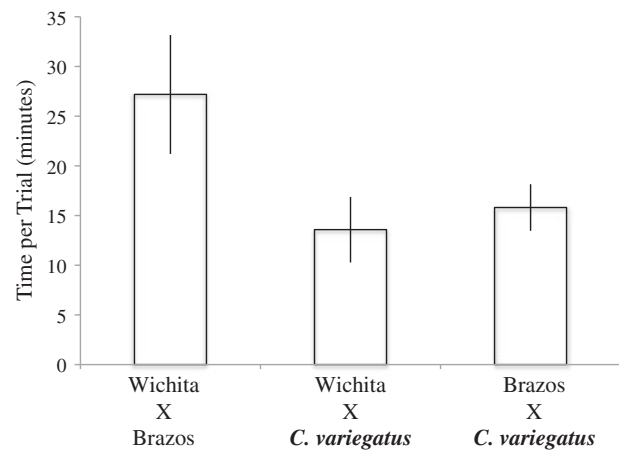
To test female mate preferences, a single, mature female was placed in the center aquarium. Females were given a 10-min acclimation time with visual dividers placed between the focal female tank and the two stimuli tanks. After the dividers were removed, data acquisition began after the female entered both interaction zones and returned to the center of the aquarium. If the female did not enter both zones within 20 min after the dividers had been removed the trial was aborted ( $n=2$ ). All entrances and exits to the interaction zones by the focal female were recorded in real time using JWatcher (version 1.0). Entering an interaction zone indicated an evaluation of that particular male, and increased evaluation of males typically results in spawning in pupfishes (Kodric-Brown 1977; Kodric-Brown 1983) and are a good predictor for female mate choice, resulting in spawning or copulation in other fishes (Ryan and Wagner 1987; Kodric-Brown 1993; Seehausen and van Alphen 1998; Walling et al. 2010). Total time spent in each zone was calculated in JWatcher, and exported for analysis.

Data in all treatments met normality assumptions according to the Kolmogorov–Smirnov, Lilliefors, and Shapiro–Wilk normality tests. A paired  $t$ -test was used to identify if there was a difference between time spent with conspecific and heterospecific males for each treatment. Strength of preference (SOP:  $(\text{Time}_{\text{conspecific}} - \text{Time}_{\text{heterospecific}}) / (\text{Time}_{\text{conspecific}} + \text{Time}_{\text{heterospecific}})$ ) was calculated for each treatment to identify the intensity of the preference for either the conspecific or heterospecific males. SOP scores range from  $-1$  to  $1$ , where positive values indicate a preference for conspecific males and negative values indicate a preference for heterospecific males. If values are close to zero, there is similar preference for both males. A  $t$ -test was used to test for differences in SOP for females in treatments with the same male types. All tests use  $\alpha=0.05$  as statistical significance.

## Results

### Male–male competition

Dominance trials varied in duration and aggression level. Fights between the males from the two populations of *C. rubrofluvialis* lasted significantly longer than fights between males from either population of *C. rubrofluvialis* and *C. variegatus* (Figure 1; ANOVA,  $F_{2, 25}=3.498$ ,  $P=0.0458$ ). No fish type (Brazos River *C. rubrofluvialis*, Wichita River *C. rubrofluvialis*, and *C. variegatus*) won more fights than the other types; there was no significant difference from an expected null 50:50 ratio for all treatments (Table 1; chi-squared test,  $\chi^2_6=8.0$ ,  $P=0.238$ ). Although size is generally a good predictor for winners in intrasexual combat



**Figure 1.** Average trial time in minutes with standard error for each treatment in dominance fights.

(Benson and Basolo 2006), the winners in these dominance trials were not significantly larger (paired  $t$ -test,  $t_{25}=0.474$ ,  $P=0.639$ ).

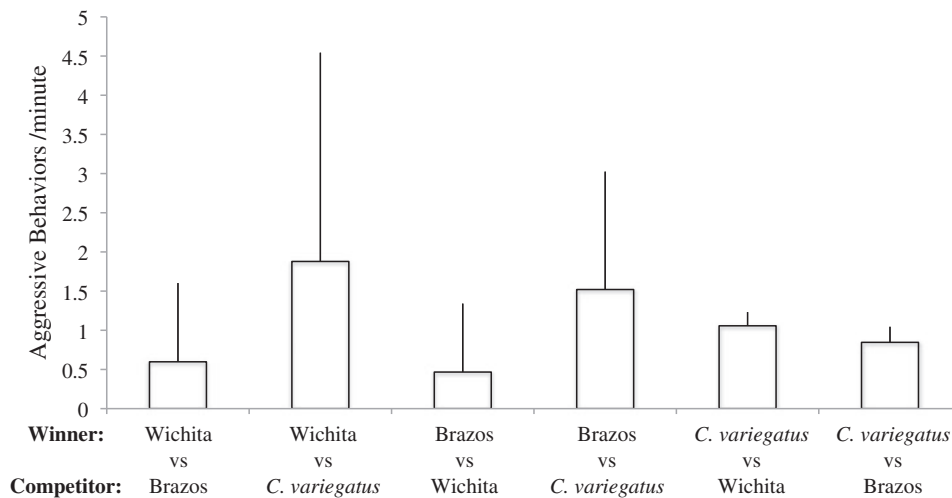
Total aggressive behaviors per minute for winners and losers of fights did not differ between the three types of males (ANOVA, Winners:  $F_{2, 25}=1.349$ ,  $P=0.278$ ; Losers:  $F_{2, 25}=1.05$ ,  $P=0.365$ ). There were no significant differences in specific aggressive behaviors for winners between the three types (ANOVA, Chases:  $F_{2, 25}=1.049$ ,  $P=0.365$ ; Bites:  $F_{2, 25}=0.641$ ,  $P=0.535$ ). However, there was a significant interaction between type and win/lose status. That is, there were significant differences when comparing winners of fights between the two species; *C. rubrofluvialis* males used significantly more aggressive behaviors to win fights against *C. variegatus*, than *C. variegatus* used to win in those fights or for winners in fights between the two populations of *C. rubrofluvialis* (Figure 2; ANOVA,  $F_{2, 25}=3.781$ ,  $P=0.037$ ).

### Female mate choice

There were no significant mate preferences by females in any treatment. Brazos River and Wichita River *C. rubrofluvialis* did not differ in the amount of time spent with conspecific males and heterospecific *C. variegatus* males (Figure 3; paired  $t$ -test, Brazos:  $t_{14}=-0.28$ ,  $P=0.78$ ; Wichita:  $t_{14}=0.16$ ,  $P=0.87$ ). *Cyprinodon variegatus* females did not spend significantly different amounts of time with conspecific males and heterospecific Brazos River *C. rubrofluvialis* males (Figure 3; paired  $t$ -test,  $t_{15}=-0.50$ ,  $P=0.63$ ). Between the two *C. rubrofluvialis* populations, Wichita River females did not differ in the amount of time spent with males of each population (Figure 3; paired  $t$ -test,  $t_{14}=1.18$ ,  $P=0.26$ ). The Strength of Preference (SOP) did not significantly differ between Brazos River *C. rubrofluvialis* females and *C. variegatus* females (Figure 4; two sample  $t$ -test,  $t_{30}=0.45$ ,  $P=0.66$ ). SOP did not differ for female Wichita River *C. rubrofluvialis* between treatments with Brazos River *C. rubrofluvialis* males and *C. variegatus* males (Figure 4; two sample  $t$ -test,  $t_{29}=0.77$ ,  $P=0.45$ ). Although there were no significant population-level preferences for conspecific and heterospecific males across treatments, individual females varied greatly in how much time they spent with particular stimuli males. Of the 61 trials tested, 15 females spent more than 75% of the time in a zone with conspecific males, and 15 spent 75% of the time in a zone with the heterospecific male. More so, 5 females spent 90% of the time with the conspecific male and 5 with the heterospecific male.

**Table 1.** Values for wins, average chases and bites per minute for each species and population winners of dominance fights across three treatments

Treatment	Winner of Fights		
	<i>Cyprinodon rubrofluviatilis</i> Wichita River	<i>Cyprinodon rubrofluviatilis</i> Brazos River	<i>Cyprinodon variegatus</i>
<i>Cyprinodon rubrofluviatilis</i> Wichita River X Brazos River	<b>Wins = 6</b> Avg. Chases/min = 0.61 ± 0.03 Avg. Bites/min = 0.30 ± 0.05	<b>Wins = 4</b> Avg. Chases/min = 0.43 ± 0.63 Avg. Bites/min = 0.10 ± 0.13	X
<i>Cyprinodon rubrofluviatilis</i> Wichita River X <i>Cyprinodon variegatus</i>	<b>Wins = 7</b> Avg. Chases/min = 1.51 ± 2.05 Avg. Bites/min = 0.36 ± 0.04	X	<b>Wins = 7</b> Avg. Chases/min = 0.90 ± 0.14 Avg. Bites/min = 0.20 ± 0.07
<i>Cyprinodon rubrofluviatilis</i> Brazos River X <i>Cyprinodon variegatus</i>	X	<b>Wins = 8</b> Avg. Chases/min = 1.37 ± 1.49 Avg. Bites/min = 0.15 ± 0.09	<b>Wins = 7</b> Avg. Chases/min = 0.69 ± 0.14 Avg. Bites/min = 0.15 ± 0.06



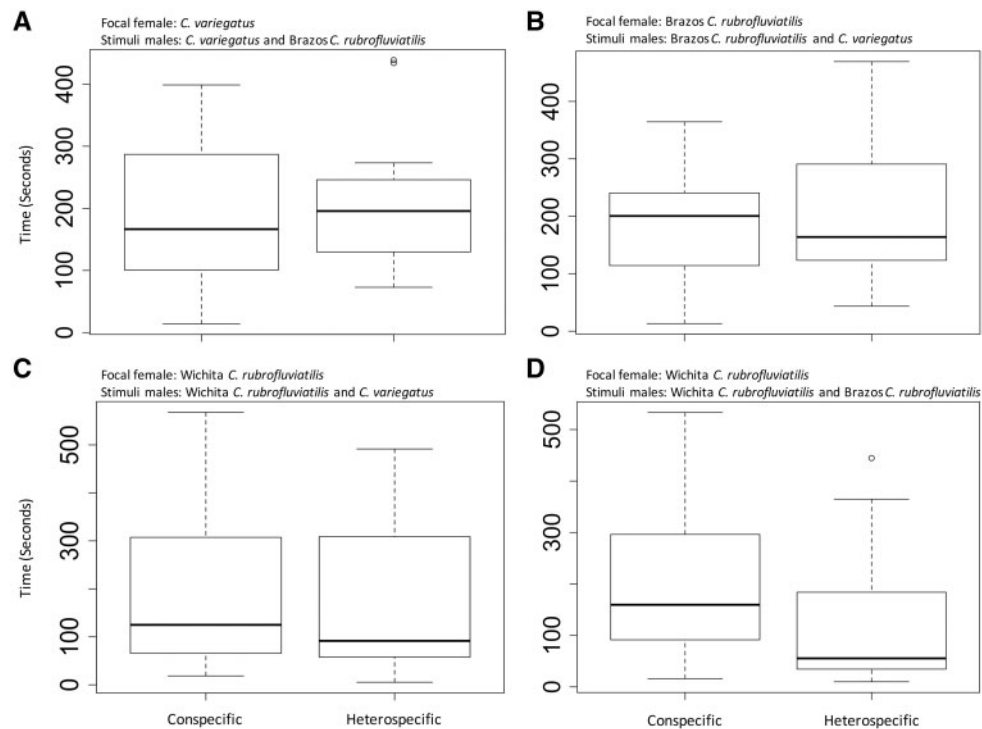
**Figure 2.** Total aggressive behaviors per minute + SE for winners of dominance fights. Winners in each treatment are shown across the top of the x-axis and the competitor is shown below winners on the x-axis.

**Discussion**

By examining male–male competition or female mate choice, we begin to elucidate the behavioral interactions upon secondary contact between the invasive *C. variegatus* and endemic *C. rubrofluviatilis*. Females of both species lack a preference for either conspecific or heterospecific males and males of both species won similar numbers of fights over breeding sites. These results suggest that reproductive isolation may not be maintained if the species come into secondary contact, and may result in widespread hybridization and introgression.

Although there was no difference in the outcome of competition between males of different species (Table 1), the aggressive behaviors needed to win access to a breeding site differed significantly between *C. rubrofluviatilis* and *C. variegatus*. *Cyprinodon rubrofluviatilis* needed a higher rate of aggression to win fights against *C. variegatus* than *C. variegatus* needed in wins versus *C. rubrofluviatilis* (Figure 2). An individual’s resource holding power (RHP) is a combination of its ability to win a competition and possess a territory, and the effort that is exerted during the duration of the contest (Parker 1974). Competing

for territories requires a large amount of energy, and it would be beneficial to win fights using the least amount of energy possible (Neat et al. 1998; Briffa and Elwood 2005; Briffa and Sneddon 2007). Ultimately, the disparity in RHP between species may favor *C. variegatus* because they do not have to exert as much energy to win territories as *C. rubrofluviatilis*. This would leave *C. variegatus* with more energy to defend territories and court females. The ability to acquire and hold a territory determines male reproductive success to a large extent (Echelle 1973; Itzkowitz 1977), indicating that these behavioral differences may have evolutionary consequences. For example, when mating is based on resources, and the males of the competitively inferior species are displaced in breeding time or habitat, reproductive isolation may occur as a by-product (Qvarnström et al. 2012). The differences in RHP may influence the ability to maintain a territory over time, providing some evidence that *C. variegatus* may be competitively superior and may contribute to displacement. Further, there could be other variables that influence competition between these species. Ecological effects often influence male–male competitive



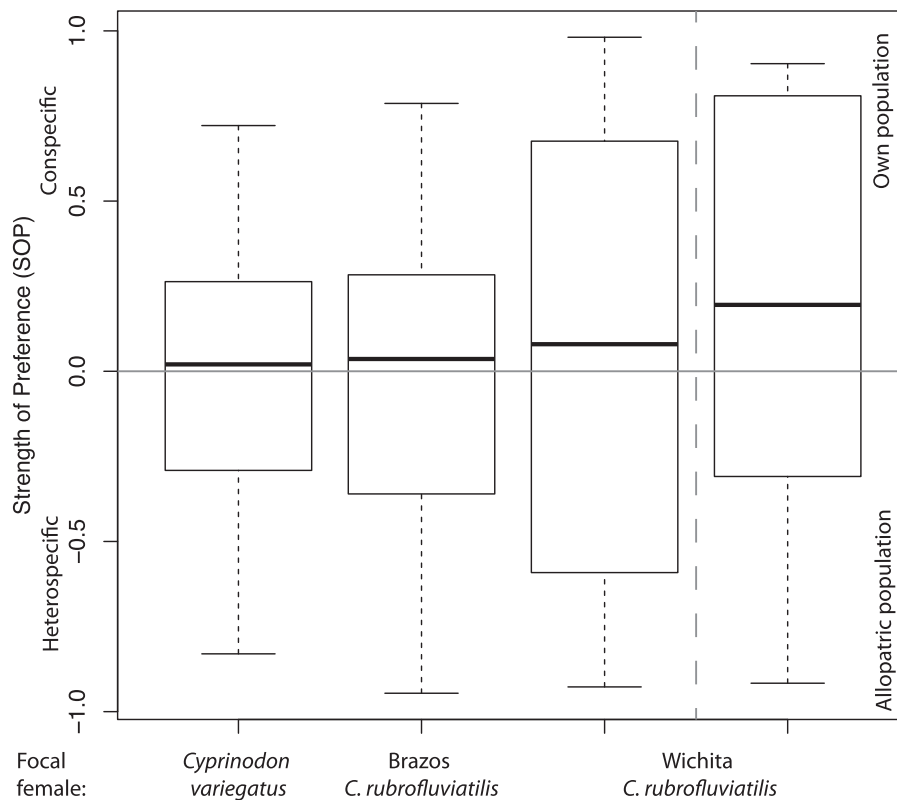
**Figure 3.** Female association time box plots for treatments, (A) Female *C. variegatus* with conspecifics and heterospecific Brazos R. *C. rubrofluvialtilis*, (B) Female Brazos R. *C. rubrofluvialtilis* with conspecifics and heterospecific *C. variegatus*, (C) Female Wichita R. *C. rubrofluvialtilis* with conspecifics and heterospecific *C. variegatus*, and (D) Female Wichita R. *C. rubrofluvialtilis* with conspecifics and Brazos R. *C. rubrofluvialtilis*.

behavior (Lackey and Boughman 2013a; Heathcote et al. 2016; Scordato 2017) and are known to influence male reproductive behavior in other species of *Cyprinodon*. Increasing the availability of habitats suitable for breeding territories can increase the number of males holding territories (Gumm et al. 2011). The population composition is also important as sex ratio and density can alter patterns of reproductive success (Gumm 2009) and the number of neighboring territorial males can influence male spawning (Leiser and Itzkowitz 2003). These factors may further exaggerate, or dampen the effects of interspecific competition, and the interaction between species interactions and ecological variables is fruitful area for future research. Additionally, although territoriality is the cornerstone of the breeding system of pupfishes, and competition over territories will have the biggest influence on reproductive success across males, territoriality is one of three reproductive tactics expressed by male pupfishes. Males expressing satellite or sneaker tactics may mediate hybridization in different ways. For example, in frogs, hybrid males are more likely to be non-calling satellites, contributing to the breakdown of reproductive isolation in a hybrid zone (Stewart et al. 2016).

Females in this study did not differentiate between males of *C. variegatus* and *C. rubrofluvialtilis* using visual cues, despite multiple morphological differences between the two species. Male *C. variegatus* are deeper bodied than *C. rubrofluvialtilis* (Page and Burr 2011; Ayers and Gumm, unpublished data) and they differ in spawning coloration in the nape and paired fins. However, stimuli males displayed breeding coloration in holding aquaria, but did not maintain the intensity of their breeding coloration when placed in the experimental aquaria. This may influence female preferences if females use color to identify mates, but Rosenfield and Kodric-Brown (2003) also found a decrease in color intensity and females still had significant preferences between male *C. variegatus* and

*C. pecosensis*. Other signals that were not examined in this study may play a role in females' evaluation of males, for example male courtship behaviors (Kodric-Brown 1989), territory defense (Rosenfield and Kodric-Brown 2003), territory quality (Kodric-Brown 1983; Ludlow et al. 2001), or olfactory cues (Strecker and Kodric-Brown 1999; Kodric-Brown and Strecker 2001). Courtship behaviors and territorial defense were controlled for by limiting physical contact between females and males, and it is unlikely that female *C. variegatus* or *C. rubrofluvialtilis* use olfactory cues for mate recognition (Gumm, unpublished data). Male size can be a factor in female mate choice in pupfishes (Draud 1996; Ludlow et al. 2001), but no evidence for the preference of larger males was detected (see "Results" section). Finally, females may use multiple cues differently, leading to high levels of individual variation in female mate preferences (Candolin 2003).

Significant female mate choice preferences at the population level were not found. However, it is important to consider that mating decisions are made at the individual level, and gene flow in this system may be maintained by the variation in female preferences for conspecific and heterospecific males (Jennions and Petrie 1997; Brooks 2002). In this experiment, individual females from each of the three groups varied in which male they spent the majority of time with; about half of the females of each type spent the majority of their time with conspecific males and about half spent the majority of time with heterospecifics. SOP was extremely variable within and across species (Figures 4 and 5). Most females had an SOP less than  $\pm 0.20$  and females with an SOP greater than  $\pm 0.80$  were evenly split in strongly preferring conspecifics and heterospecifics. This variation in identity of the preferred mate and in the strength of that preference may have important implications for the dynamics of hybridization between these two species.



**Figure 4.** SOP box plots for female preference. Values near zero indicate no preference for either type of stimulus male. Preferences for conspecific males result in an SOP value close to one, and preferences for heterospecific males or those from allopatric populations have values close to negative one. From Left to Right, plots represent (1) female *C. variegatus* preference for conspecific or heterospecific males, (2) female Brazos *C. rubrofluviatilis* preference for conspecific or heterospecific males, (3) female Wichita *C. rubrofluviatilis* preference for conspecific or heterospecific males, and (4) female Wichita *C. rubrofluviatilis* preference for conspecific males from the Wichita River or conspecific males from the Brazos River.

Comparing the mechanisms of sexual selection between allopatric populations within a species as well as between species lets us assess if patterns of behavior differ based on relatedness. In this study, females did not differ in preference behavior when choosing between a conspecific from her own population and an allopatric conspecific or between a conspecific from her own population and a heterospecific. In contrast, we found multiple aspects of male–male competition that differed between allopatric conspecific competitors and heterospecific competitors. Competitions between more distantly related males (heterospecifics) were associated with shorter times to resolve the competition compared to competitions between allopatric conspecifics. However, more aggression was needed by a male to win a competition against a more distantly related heterospecific. This bias in aggression toward distantly related heterospecifics is predicted if male–male competition plays a role in impeding gene flow and promoting divergence (Seehausen and Schluter 2004; Dijkstra and Groothuis 2011; Martin and Mendelson 2016). Our findings that male–male aggression differs between closely and distantly related groups, but female choice does not suggests that male–male competition may be the more likely mechanism to impede gene flow in this system.

Although we only found differences in male competition in this system, the two mechanisms of sexual selection are not mutually exclusive, and can act on traits independent of each other, in opposition, or in concert to strengthen selective pressure (reviewed in Wong and Candolin 2005). In some systems where male competition contributes to reproductive isolation, female choice has not

been found to play a strong role. For example, Tinghitella et al. (2015) examined both mechanisms of sexual selection in populations of threespine stickleback *Gasterosteus aculeatus* in which males do not express red coloration. They found that males that do not express red bias aggression toward red males, whereas females from all populations prefer the ancestral red coloration. Similar results have been found in colorful freshwater fishes known as darters, where male competition may play more of a role in behavioral isolation than female choice (Martin and Mendelson 2016; Moran et al. 2017). This general pattern may be common in resources based systems, like those of *Cyprinodon*, where male competition over breeding resources or nests occurs before the opportunity for female choice.

Our results are generally similar to those of other studies of interactions between endemic *Cyprinodon* spp. and invasive *C. variegatus* in that there is weak or missing sexual isolation between species (Rosenfield and Kodric-Brown 2003; Kodric-Brown and Rosenfield 2004). Our results differ from previous studies on *C. pecosensis* as we did not find male *C. variegatus* to win more competitions, or be preferred by females. There is variation in the types and extent of pre- and postmating isolation between clades of *Cyprinodon*. Specifically, two clades that have evolved in sympatry are characterized by strong reproductive isolation between species due to assortative mating driven by female mate preferences. First, an evolutionarily young, sympatric species flock of *Cyprinodon* pupfishes in Laguna Chichancanab, Mexico (Strecker 2006) shows how behavioral isolation evolves faster in sympatry than in allopatry



through asymmetries in mate choice along a genetic divergence gradient. The most genetically distinct species shows complete conspecific mate preference, the youngest species shows indiscriminate mate preference, and the intermediate species shows partial conspecific mate preference (Strecker and Kodric-Brown 2000). Another species flock of sympatric species of *Cyprinodon* in the Bahamas shows strong prezygotic isolation due to assortative mating between species, which is frequency dependent (Kodric-Brown and West 2013; West and Kodric-Brown 2015). Male-male competition has not been examined in either of these two groups. The two species flocks are examples of prezygotic isolation between sympatric species of *Cyprinodon*, however postzygotic incompatibilities and reduced hybrid fitness may be the only mechanisms that can prevent initial hybridization between allopatric *Cyprinodon* species (Cokendolpher 1980).

Biological invasions are among the most significant threats to aquatic biodiversity worldwide (Williamson 1996; Dudgeon et al. 2006), and the likelihood and pace of invasions involving hybridization are inversely related to the strength of reproductive barriers between native and non-native species (Hall et al. 2006). Our results contribute to a growing body of evidence demonstrating that the effects of human-induced environmental alterations and introductions have significant evolutionary consequences for populations and species (Seehausen et al. 2008; Hendry et al. 2008; Crispo et al. 2011; Candolin and Wong 2012; Ward and Blum 2012). The upper reaches of the Brazos River and Red River comprise the entirety of the native range of *C. rubrofluvialtilis*, and the introduction of *C. variegatus* into the Brazos River may have an important impact on the genetic integrity of the species. *Cyprinodon rubrofluvialtilis* may continue to decline in numbers due to hybridization with the invasive *C. variegatus*, potentially leading to the need for management action to avoid genetic extinction via the formation of a hybrid swarm. By examining the mechanisms that may contribute to reproductive isolation, we can further understand the process of hybridization between native and invasive species.

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