

Reinforcement of male mate preferences in sympatric killifish species *Lucania goodei* and *Lucania parva*

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Abstract Reinforcement occurs when reduced hybrid fitness leads to the evolution of a stronger prezygotic isolation. Populations sympatric with closely related species, where hybridization occurs, are predicted to have stronger mate preferences than allopatric populations. The reinforcement of male mate preference is thought to be rarer than the reinforcement of female preference, but this inference may be biased by the lack of studies on male preference. We tested male mate preferences from sympatric and allopatric populations of two closely related species of killifish: *Lucania goodei* and *Lucania parva*. We found that sympatric males had greater preferences for conspecific females than allopatric males. Furthermore, conspecific preferences in allopatric populations were weakest when these populations were geographically distant (>50 km) from those of heterospecifics. Our data suggest that reinforcement has contributed to male conspecific preference and speciation in *Lucania*.

Keywords Reinforcement · Speciation · Male mate preference · Prezygotic isolation · Hybridization

Introduction

Prezygotic isolation between species is often stronger in sympatric populations than in allopatric populations (Coyne and Orr 1989; Noor 1995). This pattern is typically attributed to reinforcement, a process by which natural selection strengthens

behavioral isolation due to fitness reductions caused by hybrid matings (Servedio and Noor 2003). This can happen via selection of stronger conspecific mate preference or selection of more distinct mating traits so that the identification of conspecifics is easier (Butlin and Ritchie 1994; Saetre et al. 1997). Originally proposed as a potentially important mechanism of speciation by Dobzhansky (1937), the support for reinforcement has varied greatly over the years. Theoretical and empirical works now uphold the idea that reinforcement can play a substantial role during divergence with gene flow (Butlin 1989; Kirkpatrick and Servedio 1999; Noor 1999; Kirkpatrick 2001; Servedio and Noor 2003; Ortiz-Barrientos et al. 2009). As the existence of reinforcement has been well established, its frequency and importance to speciation need to be determined. Theoretically, reinforcement can occur via male or female mate preferences and even when reductions in hybrid fitness are small (Kirkpatrick and Servedio 1999; Kirkpatrick 2001; Svensson et al. 2007). However, most empirical studies of reinforcement have focused on the evolution of female preferences for male traits, ignoring the contribution of male mate preferences.

Male mate preferences often involve choosing which females to court and how intensely to court them (Edward and Chapman 2011). Male courtship preferences may be especially important in polygynous mating systems where males mate with multiple females. Male mate choice evolves if there is a variation in the quality of females, and the benefit of mating with specific females is greater than the cost necessary to acquire them (Servedio and Lande 2006; Edward and Chapman 2011). Similarly, male preferences for conspecific females can also evolve via reinforcement due to the costs associated with mating with heterospecifics (Servedio 2007). Current evidence for reinforcement of male mate preference comes primarily from insects, such as damselflies, butterflies, and beetles (Waage 1979; Peterson et al. 2005; Kronforst et al. 2007; Svensson et al. 2007). However, in some bird and fish

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species, there is also evidence that males preferentially court conspecific females in sympatric populations (Ratcliffe and Grant 1983; Gabor and Ryan 2001; Albert and Schluter 2004; Magurran and Ramnarine 2005). Reinforcement of male mate preference may occur in other systems as well but has simply not been studied.

In our study, we test for reinforcement of male mate preferences in two killifish species: *Lucania goodei* and *Lucania parva*. *L. goodei* is a freshwater specialist primarily found in Florida, while *L. parva* is a euryhaline species and can be found in fresh, brackish, and marine habitats as far north as Massachusetts and as far west as central Mexico (Lee et al. 1980). In Florida, the ranges of these two species overlap, and a recent survey of Florida populations found that 15 % of *L. goodei* and 17 % of *L. parva* populations are sympatric, and most of these sympatric sites are located in fresh water (Fuller and Noa 2008). Hybridization likely occurs at sympatric sites since the morphological hybrids have been found in the field (Hubbs et al. 1943) and mitochondrial DNA haplotypes are shared between *L. goodei* and *L. parva* within sympatric drainages, indicating some introgression (R. C. Fuller, unpublished data). However, microsatellite and SNP data support the genetic distinction between these species (R. C. Fuller, unpublished data). The gene flow between species is minimized by both pre- and postzygotic isolation. Heterospecific pairs take longer to mate and produce fewer eggs than conspecific pairs (Fuller et al. 2007; Berdan and Fuller 2012). Additionally, males show preferences for conspecific females in sequential no-choice tests (Berdan and Fuller 2012). Postzygotic isolation is also present: F2 hybrid offspring have reduced viability, and some F1 hybrid males have reduced fertility (Fuller 2008). Hybrid males produced from crosses between an *L. goodei* male and an *L. parva* female have greatly decreased fertility compared to the males of the reverse hybrid cross (*L. parva* ♂ × *L. goodei* ♀) as well as the males of the two parental species (Fuller et al. 2007; Fuller 2008). The total strength of reproductive isolating barriers is estimated to be 0.997 [on a scale from 0 to 1 using the total isolation index from Ramsey et al. (2003); Berdan and Fuller (2012)].

The reductions in hybrid fitness observed between *L. goodei* and *L. parva* suggest that there is scope for reinforcement. Previous work has found no significant difference between sympatric and allopatric populations in their latency to mate when heterospecifics are paired for 30 days (Fuller et al. 2007), but this latency metric may be inappropriate given the natural history of *Lucania*. Both species are extremely iteroparous and, in the wild males, maintain territories in vegetation which females visit to lay eggs, so courtship biases may play an important role in maintaining prezygotic isolation (Fuller 2001). Male and female interactions are usually brief, and other potential mates are typically in close proximity (Arndt 1971; Fuller 2001). We have found that when male courtship is observed over shorter time periods (30 min),

males of both species preferentially court conspecific females (Berdan and Fuller 2012). Moreover, *L. goodei* males displayed greater preference for conspecifics than *L. parva* males. This asymmetry in preference is concordant with the asymmetric reductions in fertility of hybrid offspring [Berdan and Fuller 2012; see Yukilevich (2012) for a discussion of concordant asymmetries and reinforcement]. Therefore, it is possible that reinforcement has led to the evolution of preferences in *L. goodei* males.

Here, we test whether the classic signature of reinforcement (increased behavioral isolation in sympatry) occurs in *Lucania* due to male mating preferences. We observed the courtship of *L. goodei* and *L. parva* males from two allopatric and two sympatric populations. The male courtship was measured in two no-choice trials, one with a conspecific and one with a heterospecific, to determine if males preferentially court conspecific females. We predicted that if there is reinforcement of male mating preferences between populations of *L. goodei* and *L. parva*, then there would be stronger preferences for conspecific females in sympatric populations than in allopatric populations. We expected to find stronger male mating preferences for conspecifics in *L. goodei* compared to *L. parva* males, as this has been found previously (Berdan and Fuller 2012).

Methods

Experimental animals

Populations were classified as sympatric and allopatric based on extensive museum records and survey data (Fuller and Noa 2008). Additionally, for each population, we used the information in Fuller and Noa (2008) to estimate the distance to the nearest known population of the other species using coordinates of collection and path analysis along waterways in Google Earth (Google Inc, Mountain View, Canada). For sympatric populations, this distance was set to 0 km. Population and site details, classification, and distances are listed in Table 1. For each population, we collected adult males and females between May and October 2011 using dip nets and seines (St. George Sound fish were collected using a dragnet). Allopatric *L. parva* were collected from Indian River Lagoon near Titusville (Brevard County, FL, USA) and St. George Sound (Franklin County, FL, USA). Allopatric *L. goodei* were collected from Upper Bridge on Wakulla River (Wakulla County, FL, USA) and Blue Springs (Gilchrist County, FL, USA). Sympatric *L. goodei* and *L. parva* were collected from Lower Bridge on Wakulla River (Wakulla County, FL, USA) and Salt Springs (Marion County, FL, USA).

The animals were transported back to the University of Illinois where they were housed by population in 38 and 110 liter aquaria. Fish were housed in their native salinity, which was fresh water for all populations (dechlorinated city water

Table 1 Population information

| Population | Drainage | Context | Species present | Distance to other <i>Lucania</i> (km) | Site type |
|------------------|------------------|------------|--------------------------------------|---------------------------------------|-------------------|
| Indian River | Atlantic Ocean | Allopatric | <i>L. parva</i> | 13 | Coastal |
| Lower Bridge | Wakulla River | Sympatric | <i>L. goodei</i> and <i>L. parva</i> | 0 | Spring-fed stream |
| Salt Springs | St. John's River | Sympatric | <i>L. goodei</i> and <i>L. parva</i> | 0 | Lake |
| St. George Sound | Gulf of Mexico | Allopatric | <i>L. parva</i> | 52 | Coastal |
| Upper Bridge | Wakulla River | Allopatric | <i>L. goodei</i> | 6 | Spring-fed stream |
| Blue Springs | Santa Fe River | Allopatric | <i>L. goodei</i> | 120 | Spring-fed stream |

treated with Start Rite) with the exception of the two coastal populations, St. George Sound and Indian River, which were housed in salt water (reverse osmosis water with Instant Ocean® Sea Salt added to bring it to between 16 and 30 ppt). Fish were fed ad lib daily with frozen brine shrimp and flake food. The lights were maintained on a 14:10 L/D cycle. Our mating trials were conducted in fresh water, and all fish were acclimated to freshwater for at least 48 h before the start of any trials.

Mate choice trials

We assayed males from sympatric and allopatric populations for preferences for conspecifics and heterospecifics. We used sequential no-choice trials to test for male conspecific mate preference. Since males defend territories which females visit, it seems unlikely that heterospecific and conspecific females frequently visit at the same time. No-choice tests tend to be more conservative than dichotomous choice tests, so detection of the preference in a no-choice test strongly suggests that preferences exist (Houde 1997). Additionally, our trials were only 10 min, which is a biologically relevant time frame in which it is unlikely that individuals will mate simply because they have no other mating options (Fuller 2001; Foster 1967; Arndt 1971; Berdan and Fuller 2012).

We had four sets of populations: two sympatric populations from Lower Bridge and Salt Springs and two sets of allopatric populations composed of Upper Bridge *L. goodei* paired with Indian River *L. parva* and Blue Springs *L. goodei* paired with St. George Sound *L. parva* (hereafter referred to as Gulf Coast). Within each set, we tested males of both species with females of both species. Each male was observed twice—once a day on two sequential days—once with a conspecific female and once with a heterospecific female. The order in which males were presented with females (either conspecific or heterospecific) was randomized. Mate choice assays were run simultaneously for the two species (September–December 2011). Furthermore, mating preference assays for *L. goodei* and *L. parva* males from sympatric populations were conducted simultaneously. We observed four males per day.

For a given trial, a male was placed in an observation tank (38 L) at least 12 h prior to observation. Four yarn mops (two at the bottom and two floating) were placed in each observation tank for cover and as a spawning substrate. The stimulus female (either *L. goodei* or *L. parva*) was placed in a holding tank (19 L) at least 12 h prior to observation. The standard length of all fish was measured before they were placed in tanks. The observations occurred in the morning, and each female was placed into the corresponding male observation tank and was given 2 min to acclimate. After 2 min had passed, the fish were observed for 10 min.

The courtship behavior of *L. goodei* consists of a male swimming in circles around a female (circle swims) and flicking his head while in front or positioned laterally to the female (head flicks). The male approaches the underside of the female and continues performing its head flicks. The pair will then swim together, press their bodies onto the yarn mops (vegetation in the wild), and release egg and sperm to complete the spawning behavior (Foster 1967; Fuller 2001; McGhee and Travis 2010). A similar courtship is found in *L. parva*, with the exception that males only perform head flicks later in courtship when they are positioned beneath the female (Foster 1967). A live observer (O. Gregorio) recorded all the courtship behaviors displayed by the male over 10 min.

If the male and female did not emerge from hiding in the yarn mops after 4 min of observation, the observation was stopped and revisited after the remaining observations were completed (15–40 min later). After all observations on day 1 were completed, the females were removed. New females were chosen, measured, and placed into the appropriate holding tank. All females were tested with a single male. Males were removed after they had been tested with both species of female, and the observation tank was drained and refilled to prevent the transfer of chemical cues between males. We tested eight males of each species from each population. Our final data set included 47 males (eight Lower Bridge LG, five Lower Bridge LP, seven Salt Springs LG, eight Salt Springs LP, five Upper Bridge LG, five Indian River LP, five Blue Springs LG, four Gulf Coast LP; where LG equals *L. goodei* and LP equals *L. parva*). If males failed to court either female, they were removed from the data set, as their preference could not be measured. A total of ten *L. parva* males and six *L.*

goodei males failed to court any females and were removed from further analysis (an additional LG male was removed from the data set because it was in poor health). Failure to court did not differ significantly between sympatry and allopatry for either species (LP: Fisher's exact test, $P=0.25$; LG: Fisher's exact test, $P=0.08$). The average number of total courtship behaviors performed by a male ranged from 12 to 20 depending on the population (Gulf Coast/Blue Springs=19.22, Upper Bridge/Indian River=12, Lower Bridge=20.54, and Salt Springs=17.13), but the amount of courtship was not significantly different between populations ($F_{3,43}=0.63$ and $P=0.59$). Therefore, variation between populations in the motivation to court may exist, but is minor.

Statistical analysis

We calculated the proportion of each male's total courtship behavior towards the conspecific female since each male was tested with females of both species. This metric ran from 0 (all courtship directed towards the heterospecific) to 1 (all courtship directed towards the conspecific), with 0.5 indicating no preference. Using a preference score rather than the number of courtship behaviors controls for variation in courtship vigor between species and populations (Albert and Schluter 2004). Since the data ranged from 0 to 1, we analyzed it in a generalized linear model with a binomial distribution in Proc Genmod in SAS v. 9.2 (SAS Institute, Cary, NC, USA). The data were coded as the number of courtship behaviors directed towards the conspecifics over total courtship behaviors. Coding the data this way allows the least squares means to be weighted by the total number of courtship behaviors a male had performed. The main effects we tested were male species (*L. parva* and *L. goodei*), context (whether a population set was sympatric or allopatric), population set (Lower Bridge, Salt Springs, Upper Bridge/Indian River, and Blue Springs/Gulf Coast), and all interactions. Population set was nested in context since we had sympatric and allopatric population sets. Non-significant interactions were removed to simplify our model. To determine if specific populations within species were significantly different from each other, we compared least squares means from our generalized linear model. We also included the difference in body size between the conspecific and the heterospecific female as a covariate, but it was non-significant and was removed from the model ($\chi^2 < 0.01$, $P=0.98$).

Results

A higher proportion of courtship was directed towards the conspecific female when males were from sympatric populations compared to allopatric populations ($\chi_1^2=6.36$, $P=0.0117$; Table 2; Fig. 1). Based on the previous results, we had predicted that *L. goodei* males would have stronger conspecific preference

than *L. parva* males, but there was no significant difference in the strength of preference between species ($\chi_1^2=2.94$, $P=0.0865$; Table 2). However, there were significant differences in the strength of conspecific preference among populations ($\chi_2^2=16.39$, $P=0.0003$; Table 2; Fig. 1). The strength of preference for conspecifics was negatively correlated with the distance from the other *Lucania* species (Spearman rank correlation using least squares means, $r=-0.76$, $P=0.028$; Fig. 2) and not with other factors that varied among populations such as body size differences between conspecific and heterospecific females ($r=-0.33$, $P=0.42$). Gulf Coast *L. parva* and Blue Springs *L. goodei* were the allopatric populations most distant from heterospecifics and courted conspecifics significantly less than almost all other populations (the only non-significant difference was Gulf Coast vs. Indian River LP; least squares mean differences: Blue Springs LG vs. Lower Bridge LG, $Z=-3.56$ and $P=0.0004$; Blue Springs LG vs. Salt Springs LG, $Z=-2.23$ and $P=0.026$; Blue Springs LG vs. Upper Bridge LG, $Z=-2.21$ and $P=0.0274$; Gulf Coast LP vs. Lower Bridge LP, $Z=-12.43$ and $P<0.0001$; Gulf Coast LP vs. Salt Springs LP, $Z=-2.54$ and $P=0.0112$; and Gulf Coast LP vs. Indian River LP, $Z=-1.67$ and $P=0.0951$).

Discussion

The critical test of reinforcement is whether or not the proportion of courtship behavior directed towards conspecifics is affected by context (sympatry vs. allopatry). Males of both species in sympatric populations preferred to court conspecific females more than males from allopatric populations. However, there was variation among allopatric populations; males from Gulf Coast and Blue Springs were more likely to court heterospecifics than males from Upper Bridge or Indian River. This variation in preference appears to be a function of proximity of heterospecific populations. Both Gulf Coast and Blue Springs populations are more than 50 km from the heterospecifics and have weaker conspecific preferences. The Upper Bridge and Indian River populations have stronger preference for conspecific females and are much closer to heterospecific populations (less than 15 km). We classified Upper Bridge and Indian River populations as allopatric since historical records

Table 2 Results from generalized linear model for proportion of courtship directed towards the conspecifics

| Source | DF | Chi-square | <i>P</i> values |
|--------------------------|----|------------|-----------------|
| Species | 1 | 2.94 | 0.0865 |
| Context | 1 | 6.36 | 0.0117 |
| Population set (context) | 2 | 16.39 | 0.0003 |
| Species × context | 1 | 0.63 | 0.4261 |
| Error | 41 | | |

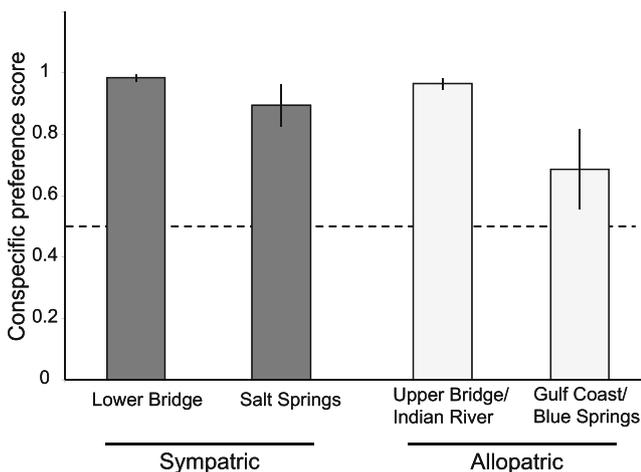


Fig. 1 Proportion of male courtship behaviors directed towards the conspecific female in each set of allopatric and sympatric populations (Lower Bridge: *L. parva*, $N=5$ and *L. goodei*, $N=8$; Salt Springs: *L. parva*, $N=8$ and *L. goodei*, $N=7$; Upper Bridge/Indian River: *L. parva*, $N=5$ and *L. goodei*, $N=5$; Gulf Coast/Blue Springs: *L. parva*, $N=4$ males and *L. goodei*, $N=5$). Means (\pm SE) are shown for both populations. The horizontal dashed line at 0.5 indicates no preference

and our own annual sampling of these populations indicate that the other species is not present (no *L. goodei* have been caught in Indian River in over 5 years and only a single *L. parva* individual has ever been caught at Upper Bridge over 10 years; Fuller et al. 2007 and R. C. Fuller, unpublished data). However, Upper Bridge is only 5.5 km upriver from Lower Bridge, and *Lucania* populations are present along the length of the river from the Lower to Upper Bridge sites. Our

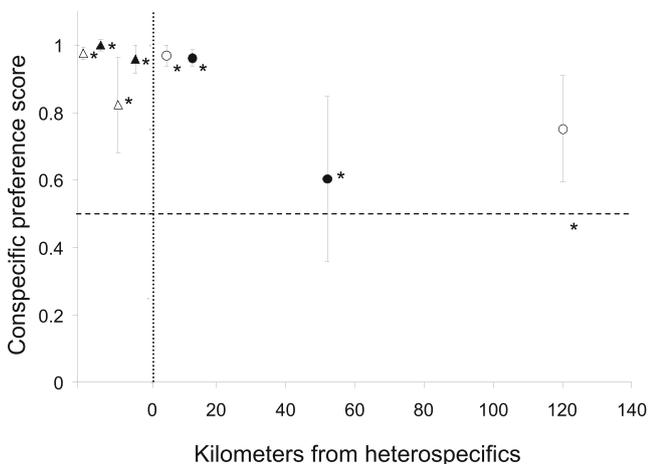


Fig. 2 Male conspecific preference vs. distance (kilometer) to the nearest heterospecific population. Means (\pm SE) are shown: filled symbols indicate *L. parva* populations; open symbols, *L. goodei*; triangles, sympatric populations; and circles, allopatric populations. Asterisks are back-transformed logit least squares means from the generalized liner model ($x = e^y / (1 + e^y)$). Sympatric populations are shown to the left of the vertical dotted line (distance=0 km) and are jittered. Populations in order from left to right are: Lower Bridge, Salt Springs, Upper Bridge, Indian River, Gulf Coast, and Blue Springs. The horizontal dashed line at 0.5 indicates no preference

Indian River population is also geographically close to both allopatric *L. goodei* populations and *L. parva* populations that co-occur with *L. goodei* on Merritt Island (Fuller and Noa 2008). The gene flow with nearby populations may have led to the transmission of conspecific preference alleles to these allopatric populations. Conspecific preference alleles might be maintained by rare encounters with members of the other species, or there may be no cost to these alleles in allopatry (Ortiz-Barrientos et al. 2009). We think it unlikely that preferences are due to imprinting since there is no parental care in these species (Fuller and Travis 2001). While we cannot definitively rule out that other forms of learning occur, the absence of congeners from Upper Bridge or Indian River and the existence of strong preferences for conspecifics suggest that preferences are transmitted via migration and gene flow rather than learning.

Our results strongly suggest that reinforcement has shaped the male courtship preferences between *L. parva* and *L. goodei*. Preferences for conspecific females are stronger in sympatry and appear to be present even in allopatric populations in close proximity to heterospecifics such as Upper Bridge and Indian River. Reinforcement of male mate preferences appears to be less common than reinforcement of female preferences, but this may be because male mate preferences are understudied (Edward and Chapman 2011) or because male preferences can be extremely context dependent (Booksmythe et al. 2011). Previous studies of increased male mate preference in sympatric populations have often focused on systems where hybridization occurs but hybrids have zero fitness (Waage 1979; Gabor and Ryan 2001; Magurran and Ramnarine 2005). Butlin (1987a, b) argued that cases where gene flow has ceased between species should be termed reproductive character displacement. This differs from reinforcement which occurs when gene flow between species is reduced but non-zero (Butlin 1987a, b). A further complication in verifying reinforcement is that selection may act directly on mate preferences. Several previous cases of strong sympatric male preferences in hybridizing populations may be due to direct selection against courting heterospecifics rather than selection against hybrids (Servedio and Noor 2003; Albert and Schluter 2004; Svensson et al. 2007). Therefore, definitive cases of reinforcement of male preferences when hybrids have reduced but with non-zero fitness are less common (Peterson et al. 2005; Kronforst et al. 2007). Our results suggest that reinforcement, rather than reproductive character displacement, has played a role in the divergence in *Lucania*. Several lines of evidence indicate that hybridization and gene flow between *L. goodei* and *L. parva* occur in sympatry (mtDNA: R. C. Fuller, unpublished data; morphology: Hubbs et al. 1943). Hybrids have reduced fitness, so selection against hybridization is likely a potent force, and there is no evidence of direct selection on male mate preferences (Fuller 2008; E. L. Berdan, unpublished data). Our study adds to growing evidence that male

mate preferences can contribute substantially to the process of speciation with gene flow.

Our data suggest a relationship between preference strength and proximity to heterospecific populations similar to previous studies in *Heliconius* butterflies and salfin mollies which have found that the strength of male conspecific preference is directly related to the distance from sympatric populations (Gabor et al. 2005; Kronforst et al. 2007). Therefore, reinforcement may frequently produce clinal effects on preference when gene flow occurs between sympatric and neighboring populations (Lemmon et al. 2004; Goldberg and Lande 2006).

In contrast to previous work, we did not find any asymmetry in male preference strength between *L. goodei* and *L. parva* (Berdan and Fuller 2012). Using methods similar to our own, Berdan and Fuller (2012) measured courtship behavior of pairs of males towards conspecific or heterospecific females and found that Upper Bridge *L. goodei* did not court *L. parva*, but Indian River *L. parva* courted *L. goodei*. In our experiment, we did not use pairs of males and instead tested a single male with a female. Both *L. goodei* and *L. parva* from our experiment displayed similar levels of conspecific preference. This suggests that the strength of *L. parva* male conspecific preference may vary based on the social context, and species differences in conspecific preference strength may occur only when other males are present. This is consistent with the work from other species showing that males display conspecific preference only in certain situations (Booksmythe et al. 2011). Additionally, within species, males can be less selective of females when other males are present due to the increased risk of competition for preferred mates (Tobler et al. 2008; Makowicz et al. 2010; Bierbach et al. 2011). An effect of social context may occur in *L. parva* because male competition is more intense in this species (Berdan and Fuller 2012), which may magnify these potential audience effects. Alternatively, context-independent rejection of heterospecifics on the part of *L. goodei* males may have been favored due to the reduced fitness of hybrid sons when these males mate with *L. parva* females (Fuller 2008). Further work is needed to discriminate between these two possibilities and to determine if audience effects exist in *Lucania*.

One alternative explanation of our results is that ecological differences between sympatry and allopatry rather than reinforcement have led to changes in male preference (Howard 1993). However, the only major environmental difference between sympatric and allopatric populations is salinity; allopatric *L. parva* populations were found in salt water, while sympatric ones came from fresh water. Previous work using a saltwater population of *L. parva* has shown that male and female conspecific mate preferences are not affected by the salinity of the mating environment (Berdan and Fuller 2012). Furthermore, crosses between freshwater and saltwater *L. parva* populations mate as quickly as crosses between two saltwater populations and provide no evidence that adaptation

to salinity causes changes to mate preferences (Kozak et al. 2012; G. M. Kozak and R. C. Fuller, unpublished data). There are no other systematic ecological differences between sympatric and allopatric populations; almost all populations differ in water depth, flow regime, wave action, pH, temperature, fish assemblage, and predators (R. C. Fuller, unpublished data; E. L. Berdan, personal observation). For example, the Wakulla River (the source of the Lower and Upper Bridge populations) is a cool, tidally influenced stream with high water flow, while Salt Springs is on Little Lake Kerr and experiences low levels of flow. Due to the lack of consistent ecological differences between sympatric and allopatric populations, we think that the pattern of male mate preference which we document is most likely due to reinforcement.

Another potential explanation of our results is that female preferences or traits differ between sympatry and allopatry which affect male preferences. We think this is unlikely for several reasons. First, males initiate courtship in *Lucania* and so courtship preferences of males are unlikely to be affected by female preferences. We did not observe males initiating courtship but then halting it due to lack of female interest. Second, we think it unlikely that the conspecific recognition cues in these species differ between sympatry and allopatry. There are no noticeable morphological differences between sympatric and allopatric fish in either species. If chemical cues are used in their courtship, these might differ between salt water and fresh water, but previous work has found this to be improbable (Berdan and Fuller 2012). It is likely that we have measured male choice in our assays rather than female preferences or traits.

In summary, our results suggest that *Lucania* males have strong conspecific mate preferences and that these preferences have likely evolved via reinforcement. Future studies should establish if there are also differences in female mate preference between sympatric and allopatric populations, allowing us to determine if both male and female preference contribute to the strong prezygotic isolation between species (Fuller et al. 2007; Berdan and Fuller 2012). Often there are asymmetries between the sexes in the strength of conspecific preference, and one sex typically contributes more to prezygotic isolation than the other (Saetre et al. 1997; Svensson et al. 2007; Kozak et al. 2009; Espinedo et al. 2010; O'Rourke and Mendelson 2010; Swenton 2011). Understanding the role of reinforcement in speciation will require detailed studies of both male and female mate preferences.

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Ethical standards All experiments comply with the current laws of the United States and were approved by the University of Illinois Institutional Animal Care and Use Committee (Protocol numbers 08183 and 09306).

Conflicts of interest The authors declare that they have no conflicts of interest.

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