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Author(s): Genevieve M. Kozak, Genevieve Roland, Clare Rankhorn, Amy Falater, Emma L. Berdan and Rebecca C. Fuller,
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Behavioral Isolation due to Cascade Reinforcement in *Lucania* Killifish

Genevieve M. Kozak,[1,*] Genevieve Roland,[1] Clare Rankhorn,[1] Amy Falater,[1] Emma L. Berdan,[2] and Rebecca C. Fuller[1]

1. Department of Animal Biology, University of Illinois, Champaign, Illinois 61820; 2. Leibniz-Institut für Evolutions- und Biodiversitätsforschung, Museum für Naturkunde, Berlin, Germany

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ABSTRACT: Reinforcement occurs when behavioral isolation is strengthened between species due to selection against hybridization in sympatry. Mate preferences and their target traits may change in sympatry as a consequence of reinforcement. This can potentially generate further behavioral isolation within species if sympatric populations evolve extreme preferences or traits that cause them to reject individuals from foreign populations as mates or be rejected as mates. This process is known as cascade reinforcement. We measured behavioral isolation between sympatric and allopatric populations of *Lucania* killifish to determine whether isolation evolves due to reinforcement between species and whether reinforcement affects preferences within species, consistent with the cascade reinforcement hypothesis. We measured mate preferences in both sexes between species (*Lucania parva* vs. *Lucania goodei*) and within species (among populations of *L. parva*). Between species, both male and female preferences for conspecifics were highest in sympatric populations. Within species, *L. parva* females from sympatric populations preferred their own native males over foreign males. Allopatric *L. parva* females and all *L. parva* males showed no preferences within species. Within species, behavioral isolation showed no association with ecological variables, such as salinity. Thus, reinforcement is a primary factor generating behavioral isolation in *Lucania* killifish, creating strong preferences in both sexes among species and leading to cascade reinforcement of female mate preference within species.

Keywords: cascade reinforcement, premating isolation, sexual selection, ecological speciation, reproductive character displacement.

Introduction

Behavioral isolation is often a key barrier to gene flow between closely related species (Coyne and Orr 2004; Boughman and Rundle 2010). Behavioral isolation can be significantly strengthened in zones of sympatry between two diverging populations through reinforcement, which occurs when hybrids have low fitness and natural selection favors changes in mating traits or preferences that allow individuals to avoid hybridization (Butlin 1989; Howard 1993; Noor 1999; Servedio 2000). This process leads to sympatric populations with stronger preferences for conspecific mates than allopatric populations (Servedio and Noor 2003; Peterson et al. 2005; Servedio 2007; Ortiz-Barrientos et al. 2009). Reinforcement can also have broader consequences for the evolution of behavioral isolation among populations within species. Strong selection on preferences and their target traits for increased species recognition in sympatric populations may carry over to mate choice within species. Individuals from sympatric populations may reject conspecific mates that originate from allopatric populations if mate preferences have become highly specific in sympatry (Hoskin et al. 2005; Pfennig and Ryan 2006). Conversely, individuals from allopatric populations may reject mates from sympatric populations if mating traits have changed (Higgie and Blows 2007, 2008). Hence, reinforcement can potentially cause a cascade of behavioral isolation in a species. First, reinforcement creates isolation between two species, and secondarily it creates isolation between sympatric and allopatric populations within a species (Ortiz-Barrientos et al. 2009). This phenomenon has been referred to as the “cascade reinforcement hypothesis” (Ortiz-Barrientos et al. 2009) and as “reproductive character displacement speciation” (Hoskin and Higgie 2010).

At present, we understand relatively little about how reinforcement causes cascades of isolation within species. There have been no theoretical studies of this process, and we currently rely on verbal models and empirical data (but see McPeek and Gavrilets 2006). Cascade reinforcement has been previously documented in several species of amphibians (Hoskin et al. 2005; Lemmon 2009; Rice and

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* Corresponding author. Present address: Department of Biology, Tufts University, Suite 4700, 200 Boston Avenue, Medford, Massachusetts 02155; e-mail: genevieve.kozak@tufts.edu.

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Pfennig 2010; Richards-Zawacki and Cummings 2011; Pfennig and Rice 2014) and insects (walking sticks [Nosil et al. 2003], rock-pool beetles [Porretta and Urbanelli 2012], and fruit flies [Jaenike et al. 2006; Higgie and Blows 2007, 2008; Dyer et al. 2014]). However, the behavioral isolation that results from cascade reinforcement differs substantially across systems. In some species, such as rock-pool beetles, sympatric populations will mate with all other sympatric populations but not with allopatric populations (Porretta and Urbanelli 2012). In other species, such as walking sticks, females from sympatric populations preferentially mate with males from their native population over males from any foreign population (Nosil et al. 2003). To date, all previous work has focused on cascade effects in female preferences and male mating traits. Therefore, it is unclear whether reinforcement between species can lead to divergence of male mate preferences within species.

Behavioral isolation may also arise through a variety of other mechanisms (Mendelson and Shaw 2012). Most notably, ecological differences between populations may cause divergence in mating cues, signals, and preferences (Dieckmann and Doebeli 1999; Schluter 2001; Servedio 2001; Otto et al. 2008; Servedio et al. 2011; Nosil 2012). Sexual selection can also cause behavioral isolation when the coevolution of mating traits and preferences leads to divergence between geographically separated populations (Ritchie 2007; M'Gonigle et al. 2012). However, sexual selection rarely generates behavioral isolation between populations without some corresponding ecological divergence (Boughman 2001; Boughman et al. 2005; Maan and Seehausen 2011; but see Mendelson et al. 2014). Ecological divergence and reinforcement are frequent and interacting contributors to behavioral isolation (Nosil et al. 2003; Pfennig and Pfennig 2010). Hence, studies of reinforcement need to consider the influence of ecology on behavioral isolation, particularly since direct ecological selection on mating preferences can potentially overwhelm indirect selection generated by hybridization in sympatric populations (Howard 1993; Servedio 2001).

In our study, we focused on testing the contribution of reinforcement to behavioral isolation between and within species of *Lucania* killifish. We tested for reinforcement of male and female preference between two closely related species: the bluefin killifish, *Lucania goodei*, and the rainwater killifish, *Lucania parva*. We also examined behavioral isolation at the within-species level. Specifically, we compared behavioral isolation between *L. parva* populations that were sympatric or allopatric with *L. goodei*. The genus *Lucania* contains three species: *L. goodei*, *L. parva*, and *L. interioris*. Molecular phylogenetic studies indicate that *L. goodei* and *L. parva* are sister taxa (Whitehead 2010), but these analyses do not include *L. interioris*, which is described as being morphologically similar to *L. parva* (Hubbs and Miller 1965). *Lucania parva* is euryhaline (i.e., found in fresh, brackish, and marine conditions) and occurs along the Atlantic Coast from Cape Cod south through the Florida Keys and throughout the Gulf Coast to the Yucatan Peninsula (Hubbs and Miller 1965). *Lucania goodei* occurs primarily in freshwater, although it occasionally is found in brackish water. *Lucania goodei* occurs primarily in Florida, with a few populations also occurring in Alabama, Georgia, and South Carolina. In Florida, *L. parva* and *L. goodei* are sympatric in 12%–19% of their populations (see below). *Lucania interioris* is confined to a single small drainage in Cuatro Ciénagas, Mexico, and is found mainly in highly alkaline waters (Miller et al. 2005). We do not consider *L. interioris* in this study.

Reinforcement requires that the species be sympatric and that hybrids have reduced fitness. Both of these conditions are met in the *L. parva*–*L. goodei* system. In Florida, several zones of sympatry occur between *L. parva* and *L. goodei*. These zones are centered in the estuarine portions of coastal rivers, where *L. goodei* from inland freshwater habitats come into contact with euryhaline *L. parva* (Fuller and Noa 2008). Hybrids between *L. parva* and *L. goodei* have been found in the lower reaches of coastal rivers (Hubbs et al. 1943), and mitochondrial DNA suggests introgression between species in sympatric locations (K. Murphy and R. C. Fuller, unpublished data). However, *L. parva*–*L. goodei* hybrids suffer reduced fitness: F_1 hybrid males in one cross direction have reduced fertility, and F_2 offspring have reduced survival at high salinities (Fuller 2008*a*). Strong behavioral isolation exists between *L. parva* and *L. goodei* in no-choice mating trials (Fuller et al. 2007; Berdan and Fuller 2012). Previous work suggests that behavioral isolation may be higher in sympatric populations. In behavioral trials, males from sympatric populations are much more likely to direct their courtship activities toward conspecific over heterospecific females than are males from allopatric populations (Gregorio et al. 2012). On the basis of the mating system, we expect that both males and females may contribute to behavioral isolation in *Lucania*. Males defend patches of aquatic vegetation that females use as egg-laying substrates. While males must decide whether to court females that enter their mating territory, females must decide whether to lay eggs in the courting male's territory and how many eggs to lay (Arndt 1971; Fuller 2001).

Ecological divergence has occurred at both among- and within-species levels in *Lucania* and may also contribute to the evolution of reproductive isolation in this genus. *Lucania goodei* is a freshwater specialist, whereas *L. parva* is euryhaline and can tolerate both low and high salinity (Fuller 2008*a*, 2008*b*; Fuller and Noa 2008; Whitehead 2010). A number of life-history traits vary both between the two species and among populations of *L. parva* as a function of salinity (Dunson and Travis 1991; Fuller 2008*a*, 2008*b*; Kozak et al. 2012). Recent comparisons of transcriptomes between the two species as well as comparisons between freshwater and saltwater populations of *L. parva* indicate that genes involved in ion transport and osmoregulation are often highly divergent between salinity environments (Kozak et al. 2014). Behavioral isolation may evolve between freshwater and saltwater populations if osmotic environments generate selection on mating traits and/or preferences. However, our work to date does not provide strong support for this hypothesis. Between species, we find similar levels of behavioral isolation regardless of whether mating assays are conducted in freshwater or saltwater, suggesting that isolation does not change in response to salinity (Berdan and Fuller 2012). However, adaptation to salinity may lead to genetic changes in mating traits and preferences between *L. parva* and *L. goodei* that need not be plastic with respect to salinity. This can be tested by comparing behavioral isolation between population pairs inhabiting the same ecological conditions (e.g., behavioral isolation between two freshwater populations) with behavioral isolation between population pairs inhabiting different ecological conditions (e.g., behavioral isolation between freshwater-saltwater population pairs). Mating in no-choice assays occurs readily between freshwater and saltwater populations (Kozak et al. 2012). However, individuals often display preferences in dichotomous-choice tests that are not observed in no-choice tests (Booksmythe et al. 2011). Therefore, we tested *L. parva* populations from both freshwater and saltwater to ask whether salinity, rather than sympatry, contributes to the evolution of mate preferences among populations within *L. parva*.

In our study, we conducted dichotomous mate preference tests for both males and females. We tested whether sympatric female *Lucania* have preferences for conspecifics over heterospecifics. We also tested sympatric males and directly compared the strength of male and female preferences to test the role played by reinforcement in generating preferences in both sexes. To test for cascade reinforcement within *L. parva*, we compared populations sympatric or allopatric with *L. goodei* and asked whether males or females had preferences for mates from their native population over mates from foreign populations.

We predicted that if reinforcement contributes to behavioral isolation between species, then we would find strong preferences for conspecifics only in sympatric populations. If cascade reinforcement is occurring, we predicted that sympatric populations would also have strong preferences for native over foreign mates. We also tested the role played by ecological adaptation by comparing isolation between freshwater and saltwater *L. parva* populations. If ecological adaptation to different salinities has contributed to the evolution of behavioral isolation, then behavioral isolation should be high between freshwater and saltwater populations but not between populations inhabiting similar salinities. If sexual selection in the absence of reinforcement or ecological adaptation has contributed to behavioral isolation, then all individuals from all populations should prefer native mates over foreign mates regardless of whether *L. goodei* is present. Preference for native mates in all populations could also be generated if gene flow between sympatry and allopatry has erased the signature of (cascade) reinforcement. By measuring preferences within and between species, we can determine whether the factors generating behavioral isolation within *L. parva* are the same as those contributing to isolation between *L. parva* and *L. goodei*.

Methods

Population type (sympatric, allopatric) was determined using museum records and our own survey data (Fuller and Noa 2008; Gregorio et al. 2012; see table 1). The extent of sympatry between *Lucania parva* and *Lucania goodei* differs between river drainages due to variability in *L. parva*'s colonization of freshwater habitats. On the Atlantic Coast of Florida, sympatry extends throughout the St. John's River drainage. Freshwater sympatric *L. parva*

Table 1: Population information

ID	Population	Salinity (ppt)	Context	Body of water
1	Gulf Coast *Lucania parva*	Salt (32)	Allopatric	St. George Sound
2	Blue Springs *Lucania goodei*	Fresh (.01)	Allopatric	Sante Fe–Suwannee River
3	Lower Bridge *L. goodei*	Fresh (.2)	Sympatric	Wakulla River
4	Lower Bridge *L. parva*	Fresh (.2)	Sympatric	Wakulla River
5	St. John's River *L. parva*	Fresh (.6)	Sympatric	St. John's River
6	Indian River *L. parva*	Salt (32)	Sympatric[a]	Indian River Lagoon
7	Bolivar Peninsula *L. parva*	Salt (16–32)	Allopatric	Galveston Bay, Texas

Note: Numbers correspond to population labels in figure 1.

[a] Eight kilometers from sympatric.

were collected from the St. John's River in Putnam County near Palatka (fig. 1). Saltwater sympatric *L. parva* were collected from Indian River Lagoon (Brevard County) on the Atlantic Coast. We classified Indian River as sympatric a priori because, although there were no *L. goodei* at the collection site, this population was close (~8 km) to a number of *L. goodei* populations on Merritt Island and sympatric populations in the nearby St. John's River (Fuller and Noa 2008). In previous work, the Indian River population was behaviorally indistinguishable from other sympatric populations (Gregorio et al. 2012). On the Gulf Coast, zones of sympatry are limited to the coastal portions of rivers. We sampled freshwater sympatric *L. parva* and *L. goodei* from Lower Bridge in the lower reaches of the Wakulla River (Wakulla County). Distantly allopatric *L. goodei* were sampled from Blue Springs in the Sante Fe River, an inland branch of the Suwannee River more than 120 km from coastal *L. parva* populations (Gregorio et al. 2012). Distantly allopatric saltwater *L. parva* were collected from St. George Sound off the Gulf Coast, a site more than 50 km from inland *L. goodei* populations. Fish from all populations were collected with dip nets and seines in 2011 or 2012, with the exception of the St. George Sound (Gulf Coast) fish, which were collected using a drag net.

Fish were brought back to the University of Illinois, where they were maintained at their native salinity in stock tanks. All mate-choice tests occurred in freshwater with salinity between 0 and 0.5 ppt, as previous work suggested that the salinity of the mating environment has no effect on behavioral isolation (Berdan and Fuller 2012). Before being placed in testing tanks, fish from saltwater populations were transitioned to an intermediate salinity (10 ppt) for several days. Freshwater was created from city water treated with the dechlorinating agent API Tap Water Conditioner (Mars Fishcare, Chalfont, PA) and alkaline buffer (Seachem Laboratories, Madison, GA). Saltwater was created by adding Instant Ocean Sea Salt (Spectrum Brands, Atlanta, GA) to reverse-osmosis (RO) water. RO water was created using a four-stage barracuda RO/DI unit (Aqua Engineering and Equipment, Winter Park, FL). The fish were kept on a light cycle of 14L∶10D and fed a mixture of frozen brine shrimp and flake food ad lib. We conducted female trials between May and August 2012 and male trials between September and November 2012.

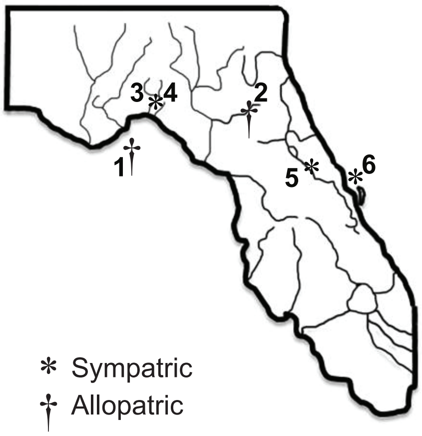

Figure 1: *Lucania* study populations in Florida. Shown is a map of Florida major river drainages labeled with numbers corresponding to table 1. Daggers indicate allopatric populations, and asterisks indicate sympatric populations. 1 = Gulf Coast *Lucania parva*, St. George Sound, Gulf of Mexico (allopatric); 2 = Blue Springs *Lucania goodei*, Rum Island Park, Santa Fe River, Columbia County (allopatric); 3 = Lower Bridge *L. goodei* Wakulla River, Wakulla County (sympatric); 4 = Lower Bridge *L. parva*, Wakulla River, Wakulla County (sympatric); 5 = St. John's River *L. parva*, Putnam County (sympatric); 6 = Indian River Lagoon *L. parva*, Brevard County, Atlantic Ocean (sympatric).

Dichotomous-Choice Behavioral Trials

We used the same behavioral assays to measure mate choice both between species and among populations of *L. parva*. All behavioral trials were performed in the morning and early afternoon (0700–1400 hours). For female trials, stimulus males were introduced into 38-L testing tanks that were visually isolated from other tanks the evening before the test. There was no water exchange between tanks. Two males were placed into each tank, one on each side of a mesh divider that separated each tank into two halves. The divider ensured that males established territories on only their side of the tank. Female fish were housed for at least 16 h with other females from their population in the same room where trials took place. Housing females in the absence of males (for short periods of time) increases the probability that they have mature eggs and are ready to mate. All fish were fed in the morning at least 15 min before behavioral trials began.

Before each trial, we placed stimulus males in mesh wire cages (90 mm long × 90 mm wide × 85 mm tall; mesh size = 1.5 mm^2) that allowed transmission of visual and olfactory cues of the males but prevented male-female contact. At the beginning of the trial, we suspended the mesh boxes near the surface of the water in the back corner of each side of the tank and removed the divider. We introduced the focal female to the center of the tank, where a yarn mop provided shelter. The female was then given 2–5 min to acclimate. Once the female was exploring the tank, she was considered acclimated, and the male cages were lowered to rest on the bottom. The female could then choose to associate with either male. Using stopwatches, we measured the time spent by the female within one body length of each cage over a 10-min (600-s) period. After the trial, standard lengths of males and females were measured. Only trials in which the female visited both male cages (during acclimation or the trial itself) were included in our data set.

Male trials were similar to female trials except that a single male was introduced to the tank the evening before to establish a territory, and females were introduced into cages immediately before the trial. Males and females were then given 2–5 min to acclimate to this setup before behavioral measurements began. An observer used a voice recorder to note time spent within one body length of each cage as well as the number of courtship bouts (headflicks) performed when males were in each preference zone. However, we excluded headflicks from our statistical analyses due to the low number of trials in which males unambiguously directed headflicks toward a female (between species: 10 of 40 males; within *L. parva*: 14 of 50 males).

Focal fish were never reused within an experiment. However, focal fish were sometimes reused between experiments; a few individuals were used once in the between-species reinforcement experiment as well as once in the cascade reinforcement experiment within *L. parva*. These fish were reused on different days. Stimulus fish (i.e., the objects of the mating preferences) were occasionally reused after a week in stock tanks. Stimulus animals were never used twice in the same treatment on the same day; a small number of stimulus males (four males) were used twice in different treatments on the same day for the cascade experiment within *L. parva.*

Conspecific Mate Preference between Species

Reinforcement predicts increased preference for conspecifics in sympatric populations. To test this prediction for females, we compared the mate preferences of sympatric females (Lower Bridge *L. parva* and *L. goodei*) and allopatric females (Gulf Coast *L. parva*, Blue Springs *L. goodei*). Our experimental design considered the effect of context (allopatry or sympatry) and species (*L. goodei* or *L. parva*), resulting in a 2 × 2 design with four levels (1: sympatric female *L. goodei* choosing between sympatric males; 2: sympatric female *L. parva* choosing between sympatric males; 3: allopatric female *L. goodei* choosing between allopatric males; and 4: allopatric female *L. parva* choosing between allopatric males; see table 2). We performed a total of 12 trials of each type. The location of the conspecific male (right or left side of the tank) was alternated between trials. To test for reinforcement of male preference, we used the same experimental design and examined the effects of context (sympatry, allopatry) and species (*L. goodei*, *L. parva*) on male preference for conspecific females. Again, this resulted in a 2 × 2 design with four levels (see above). We tested 10 males for each of our four combinations.

We calculated both absolute and scaled preferences. Absolute preference was the difference (in seconds) in the amount of time spent with the conspecific mate minus the amount of time spent with the heterospecific mate. We calculated scaled preference as the amount of time spent with the conspecific mate minus the time spent with the heterospecific mate divided by the total amount of time spent in any preference zone (time with conspecific mate plus time with heterospecific mate; Albert and Schluter 2004). These two metrics produced nearly identical qualitative patterns.

All statistical analyses were conducted in R (ver. 3.01; R Development Core Team 2014). For all tests, effect sizes (Cohen's *d*) are listed in addition to *F* statistics and were calculated using the compute.es package in R. We first analyzed each sex separately (table 3, pts. A, B). We used ANCOVA to test the effects of context (sympatric, allopatric), species identity of the focal individual (*L. goodei*, *L. parva*), body size difference (between the conspecific and the heterospecific mate), and all possible interactions. Body size and its interactions with the main effects were not significant in either the male or the female analysis (all $P > .10$) and were removed to simplify the models. To test for differences between the sexes in the strength of preference, we combined male and female data. We performed an ANOVA including sex and its interactions with other factors in the model (table 3, pt. C). Mean preferences (± 1 SE) and significance from 0 from the ANOVAs are presented throughout.

Finally, we performed an additional assay to determine whether association time was actually a manifestation of social partner preference rather than a measure of female mating preference. Both species are known to school in the wild with conspecifics (Arndt 1971). Twelve trials were performed in which females were given a choice between associating with either a female *L. goodei* or a female *L. parva*. We used females only from the sympatric (Lower Bridge) population because sympatric females of both species showed elevated levels of preference for conspecifics (see below). Six female *L. goodei* and six female *L. parva* were assayed. The assay was identical to that used for female choice between males with the exception that females were housed in the mesh boxes. We measured social partner preference as the amount of time spent with the conspecific female minus that spent with the heterospecific female. We used a one-way *t*-test to test the hypothesis that preference for conspecifics was significantly greater than 0.

Mate Preferences within Lucania parva

Cascade reinforcement predicts systematic differences between allopatric and sympatric populations. We compared

Table 2: Mate-choice trial types

Trial type	Focal individual	Focal context	Conspecific population	Heterospecific population	Salinity	No. female trials	No. male trials
Between species:							
1 vs. 2	Gulf Coast *Lucania parva*	Allopatric	Gulf Coast *L. parva*	Blue Springs *Lucania goodei*	Freshwater-saltwater	12	10
2 vs. 1	Blue Springs *L. goodei*	Allopatric	Blue Springs *L. goodei*	Gulf Coast *L. parva*	Freshwater-saltwater	12	10
3 vs. 4	Lower Bridge *L. goodei*	Sympatric	Lower Bridge *L. goodei*	Lower Bridge *L. parva*	Freshwater-freshwater	12	10
4 vs. 3	Lower Bridge *L. parva*	Sympatric	Lower Bridge *L. parva*	Lower Bridge *L. goodei*	Freshwater-freshwater	12	10
	Focal individual	Focal context	Native population	Foreign population	Salinity	No. female trials	No. male trials
Between *L. parva* populations:							
1 vs. 6	Gulf Coast *L. parva*	Allopatric	Gulf Coast *L. parva*	Indian River *L. parva*	Saltwater-saltwater	10	10
1 vs. 5	Gulf Coast *L. parva*	Allopatric	Gulf Coast *L. parva*	St. John's *L. parva*	Freshwater-saltwater	10	0
6 vs. 1	Indian River *L. parva*	Sympatric	Indian River *L. parva*	Gulf Coast *L. parva*	Saltwater-saltwater	10	10
6 vs. 5	Indian River *L. parva*	Sympatric	Indian River *L. parva*	St. John's *L. parva*	Freshwater-saltwater	10	0
6 vs. 4	Indian River *L. parva*	Sympatric	Indian River *L. parva*	Lower Bridge *L. parva*	Freshwater-saltwater	10	0
5 vs. 4	St. John's *L. parva*	Sympatric	St. John's *L. parva*	Lower Bridge *L. parva*	Freshwater-freshwater	11	10
5 vs. 6	St. John's *L. parva*	Sympatric	St. John's *L. parva*	Indian River *L. parva*	Freshwater-saltwater	10	0
5 vs. 1	St. John's *L. parva*	Sympatric	St. John's *L. parva*	Gulf Coast *L. parva*	Freshwater-saltwater	9	0
4 vs. 5	Lower Bridge *L. parva*	Sympatric	Lower Bridge *L. parva*	St. John's *L. parva*	Freshwater-freshwater	11	10
4 vs. 6	Lower Bridge *L. parva*	Sympatric	Lower Bridge *L. parva*	Indian River *L. parva*	Freshwater-saltwater	11	10

Note: For female tests, the focal individual was a female, and the conspecific and heterospecific individuals were males. For male tests, the focal individual was a male, and the conspecific and heterospecific individuals were females. Trial type uses population numbers from figure 1, with focal population listed first.

Table 3: ANOVA and effect sizes for between-species preferences

Factor	df	Absolute preference				Scaled preference			
		MS	*F*	Cohen's *d*	*P*	MS	*F*	Cohen's *d*	*P*
A. Female mate preference between species:									
Context (sympatric, allopatric)	1	265,944	11.69	.95	.001	3.20	19.89	1.21	<.0001
Species (*Lucania goodei*, *Lucania parva*)	1	88,311	3.88	.55	.06	.28	1.76	.36	.19
Context × species	1	3,997	.17	. . .	.68	.27	1.67	. . .	.20
Error	44	22,749				.16			
B. Male mate preference between species:									
Context (sympatric, allopatric)	1	398,801	7.38	.85	.01	1.40	4.44	.67	.04
Species (*L. goodei*, *L. parva*)	1	3,098	.057	.08	.81	.09	.29	.17	.59
Context × species	1	4,368	.081	. . .	.77	.03	.098	. . .	.76
Error	36	54,004				.32			
C. Combined male and female mate preference between species:									
Context (sympatric, allopatric)	1	650,652	17.67	.88	<.0001	4.48	19.51	.92	<.0001
Sex (male, female)	1	5,940	.16	.08	.69	.0038	.01	.02	.90
Species (*L. goodei*, *L. parva*)	1	66,048	1.79	.28	.18	.36	1.40	.25	.21
Context × sex	1	14,093	.38	. . .	.53	.11	.51	. . .	.49
Context × species	1	5	<.01	. . .	.99	.070	.27	. . .	.58
Sex × species	1	25,360	.69	. . .	.40	.018	.10	. . .	.77
Context × sex × species	1	8,361	.23	. . .	.64	.23	.91	. . .	.32
Error	80	36,813				.23			

the preferences of female *L. parva* (for native vs. foreign males) between a distantly allopatric population (Gulf Coast) and three sympatric populations (Indian River, St. John's, Lower Bridge). Due to logistical constraints, we could not measure female preference for all possible population pairs. Rather, we chose to examine a subset, testing each female population with least two different foreign populations (table 2; 10 population pairs in total).

In females, these same assays could be used simultaneously to test the hypothesis that divergent ecological selection leads to increased behavioral isolation. For some trials, females were given the choice between native and foreign males when the foreign male was from a similar salinity environment. In other trials, females were given the choice between native and foreign males when the foreign male was from a different salinity environment. We predicted that behavioral isolation might be stronger between populations that occur in different salinities versus those occurring in similar salinity conditions. Our two freshwater populations were Lower Bridge and St. John's; our two saltwater populations were Gulf Coast and Indian River. We tested population sets reciprocally: focal females from population A choosing between males from populations A and B, and focal females from population B choosing between males from populations A and B. We tested three reciprocal freshwater-saltwater sets (St. John's–Gulf Coast, Lower Bridge–Indian River, St. John's–Indian River), one reciprocal saltwater-saltwater set (Gulf Coast–Indian River), and one freshwater-freshwater set (Lower Bridge–St. John's). We tested 9–11 focal females per unique population pair (10 pairs in total: 4 same salinity, 6 different salinities), resulting in 102 females in total (see table 2).

We used similar methods to compare male preferences between allopatric and sympatric populations, but we used fewer population pairs (and not all population pairs were tested reciprocally). We performed reciprocal trials for the two freshwater populations (Lower Bridge–St. John's) and the two saltwater populations (Gulf Coast–Indian River). We performed one set of nonreciprocal trials between a freshwater-saltwater population pair: Lower Bridge males choosing between Lower Bridge (freshwater) versus Indian River (saltwater) females. We tested 10 males per unique population combination for a total of 50 males (table 2).

Our goal was to determine whether preferences for native over foreign mates existed within *L. parva* and whether they differed between populations. For each sex, we calculated absolute preference as the difference in the amount of time spent with the native population mate minus the amount of time spent with the foreign population mate. Again, we calculated scaled preference as the absolute preference divided by the total time spent with any mate. Body size difference was calculated as the difference in standard length between the potential mate from the individual's native population versus the foreign population. We used ANCOVA to test for differences in preference (tables A1, A2; tables A1–A5 are available online). For female analyses, we included three factors in our model: focal population (Gulf Coast, Indian River, Lower Bridge, St. John's), foreign population salinity (same, different) nested within focal population, and body size difference of potential mates (a continuous variable). We tested for differences between allopatric and sympatric populations using the post hoc Tukey corrected *t*-test. To test for an effect of sympatry on male preference, we analyzed the preference of all male populations (when the foreign population was from the same salinity) with male population as a factor in the model and body size as a covariate. Only Lower Bridge males were tested using foreign females from both salinity environments. Hence, we tested for an effect of salinity by analyzing the preference of Lower Bridge males with foreign population salinity as a factor in the model and body size as a covariate. If body size and/or its interactions with population were not significant ($P < .10$), then they were removed to simplify the models.

To compare the strength of preference between the sexes, we tested for differences in preference between males and females in all population pairs in which both males and females were tested (table A3). We included sex, context (allopatric, sympatric), and the interaction between sex and context as factors in the model with body size difference as a covariate.

Finally, we also performed an egg-laying assay of total behavioral isolation between populations of *L. parva* to determine whether the female preferences we documented with our behavioral assays actually reflected mating behavior when animals had the opportunity to spawn. After female *L. parva* behavioral trials, we replaced the dividers in the tanks to separate the males. Females were paired with each male separately for the egg-laying assay. A female was left to freely interact with one of the males (chosen at random) on his side of the tank for 2 h, and the number of eggs laid over that time was counted. The female was then moved to the other male's side of the tank, and again the number of eggs she laid with him over 2 h was counted. This assay is dependent on both female and male preferences since females will lay eggs only if males court them (Arndt 1971). Thus, the number of eggs laid is a measure of total behavioral isolation. A total of 62 of 102 females laid at least one egg with one of the males and were included in the sample; 35 of these females saw males from their native population first, and 27 females saw males from foreign populations first. We measured behavioral isolation as the difference in the number of eggs laid with males from their native population versus males from a foreign population for the 62 females that produced at least one egg. These data were analyzed in an ANOVA with focal population (Gulf Coast, Indian River, St. John's, Lower Bridge) and the foreign male's salinity nested within focal population (table A4).

Additional Preference Assay

The within–*L. parva* mate-preference assays described above were used to measure population-specific preferences for one allopatric population and three sympatric populations. To determine whether the patterns were robust, we examined mate preferences in *L. parva* from a second allopatric population collected from Bolivar Peninsula near Galveston, Texas (table 1; described in detail in Kozak et al. 2014). This allopatric Texas population of *L. parva* is more than 800 km from the western range limit of *L. goodei* (in Alabama and Florida; Fuller and Noa 2008). We compared mating preferences of Texas (TX) fish to mating preferences from the sympatric Indian River (IR) population. We used a no-choice egg-laying assay where we paired females with either a native or a foreign male in large 101-L aquariums. We conducted eight replicates of each cross type (each female by male combination: IR × IR, IR × TX, TX × TX, TX × IR) that were established in a temperature-controlled greenhouse in September 2011. Eggs were collected from yarn mops every 3 days for 14 days total. We analyzed the number of eggs produced in an ANOVA with cross type as a factor.

Male Traits

To quantify differences in male morphology between *L. parva* populations, five male *L. parva* from each of the four populations were anesthetized in a dilute solution of MS-222 and photographed on a 1-mm grid with dorsal and anal fins fully extended. Six male *L. goodei* collected from the Upper Bridge site in the Wakulla River (5 km upstream from the sympatric Lower Bridge site) were also measured using the same methods. Body length and anal fin area in millimeters were measured using ImageJ. We performed one-way ANOVAs comparing standard length and anal fin area among populations. We also performed an ANCOVA on anal fin area using standard length as a covariate and population as a fixed effect.

Results

Conspecific Mate Preference between Species

There was strong evidence for reinforcement of female preference between species. In both species, females from sympatric populations preferred conspecific over heterospecific males, whereas females from allopatric populations did not have a preference for conspecifics (fig. 2; table 3, pt. A). Both sympatric female *Lucania goodei* and female *Lucania parva* spent significantly more time with conspecific males, and there was no significant difference in the strength of female preference between sympatric *L. goodei* and *L. parva*. Allopatric Gulf Coast female *L. parva* showed no preference for males of either species. Allopatric Blue Springs female *L. goodei* tended to prefer heterospecific males, although this was not significant. These trials captured female mate preference and not social partner preference because sympatric females did not prefer to associate with females of their own species (paired *t*-test: $t_{11} = 1.32$, $P = .21$; fig. A1; figs. A1–A5 are available online).

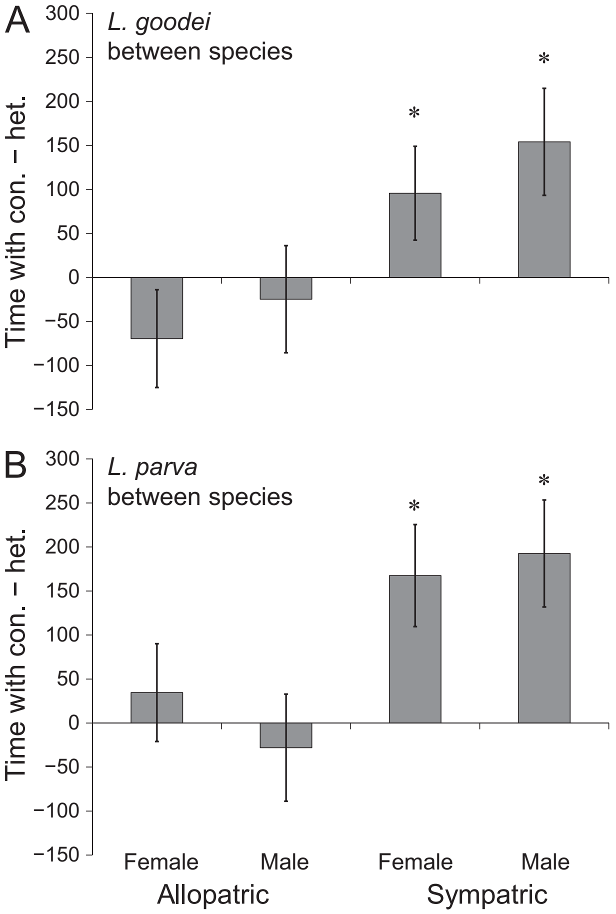

Figure 2: Mate preferences between species in male and female *Lucania goodei* and *Lucania parva*. *A*, *Lucania goodei* mean (±1 SE) preference for their own species (time spent with conspecific [con.] mate minus time spent with heterospecific [het.] mate). *B*, *Lucania parva* preference for mates of their own species. Significance of preference difference from 0 is indicated by asterisks ($P < .05$).

Similarly, males from sympatric populations also spent significantly more time with conspecific mates than did males from allopatric populations (fig. 2; table 3, pt. B). Sympatric males of both species showed a significant preference for conspecific females. The strength of preferences did not differ between sympatric populations of the two species. Allopatric males showed no preferences for conspecific females.

Males and females did not differ significantly in the amount of time they spent with conspecifics from either context or either species (table 3, pt. C; all interactions with sex were nonsignificant). In sympatry, males and females of both species had equally strong preferences (fig. 2). Our results were similar if we used a scaled preference measure. Again, we found that sympatric males and females showed stronger preferences than allopatric individuals (table 3, pts. A–C).

Mate Preferences within Lucania parva

We found that female *L. parva* preferred males from their native population over foreign mates only when females originated from sympatric populations (fig. 3). There were significant differences in preference among female populations (table A1). Females from sympatric populations had strong preferences for mates from their native population, while females from our allopatric population showed no preference (i.e., preference was significantly greater than 0 for all sympatric populations but not for the allopatric population). Preferences for native mates were nearly three times greater in sympatric than in allopatric populations (all pairwise comparisons between sympatric and allopatric populations: $t_{94} > 4.3$, Tukey corrected $P < .0002$; *t*-test assuming unequal variance: sympatry vs. allopatry $t_{47.12} = 8.4$, $P < .0001$). Again, using scaled preferences did not change our results (all pairwise comparisons between sympatric and allopatric populations: $t_{94} > 4.4$, Tukey corrected $P < .0002$; *t*-test assuming unequal variance: sympatry vs. allopatry $t_{65.086} = 9.56$, $P < .0001$; table A1).

Ecological differences in salinity had little effect on female preference. Figure 3 shows the absolute preference of females for native males over foreign males from a similar salinity environment (white bars) and a different salinity environment (gray bars). Preferences for native males did not depend on whether foreign populations were found in similar or different salinity conditions (table A1). Only St. John's females showed increased preference for native males when foreign males came from a different salinity; Lower Bridge, Indian River, and Gulf Coast females had equivalent preferences for foreign males across salinities. Finally, our main finding of increased female preference for native males in populations sympatric with *L. goodei* remained even when the analysis was limited to females that saw only males from the same salinity environment (table A1).

Male *L. parva* showed no preference for females from their native population over foreign populations from the same salinity (fig. 4). Strength of preference did not differ

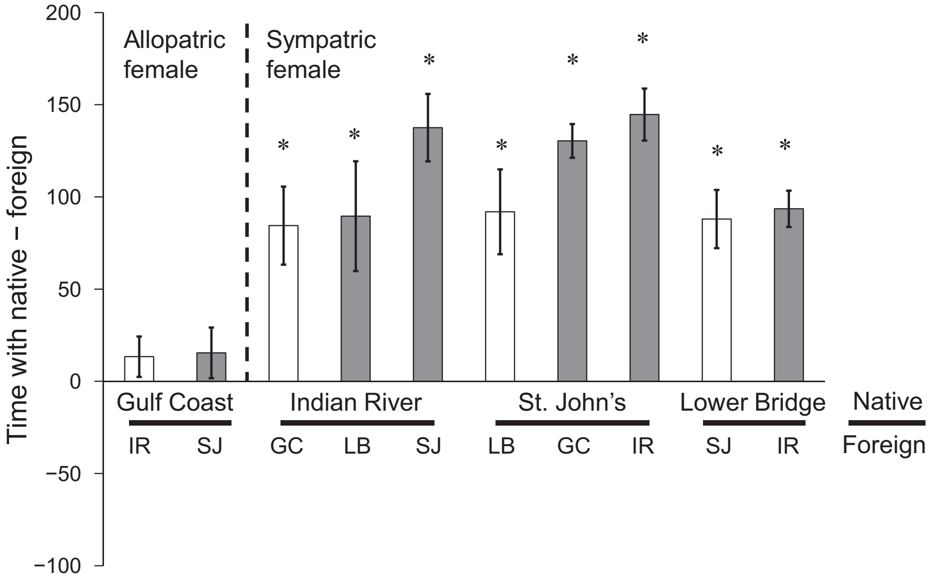

Figure 3: Female *Lucania parva* preference for mates of their native population over mates from foreign *L. parva* populations. Mean (± 1 SE) preference for native mates is shown. Native population of focal individual is listed above each line, and foreign mate population is listed below (GC = Gulf Coast; IR = Indian River; SJ = St. John's; LB = Lower Bridge). White bars indicate when both native and foreign *L. parva* populations are from the same salinity, and gray bars indicate populations from different salinities. Gulf Coast *L. parva* are allopatric; all other populations are sympatric with *Lucania goodei*. Significance of preference difference from 0 is indicated by asterisks ($P < .05$).

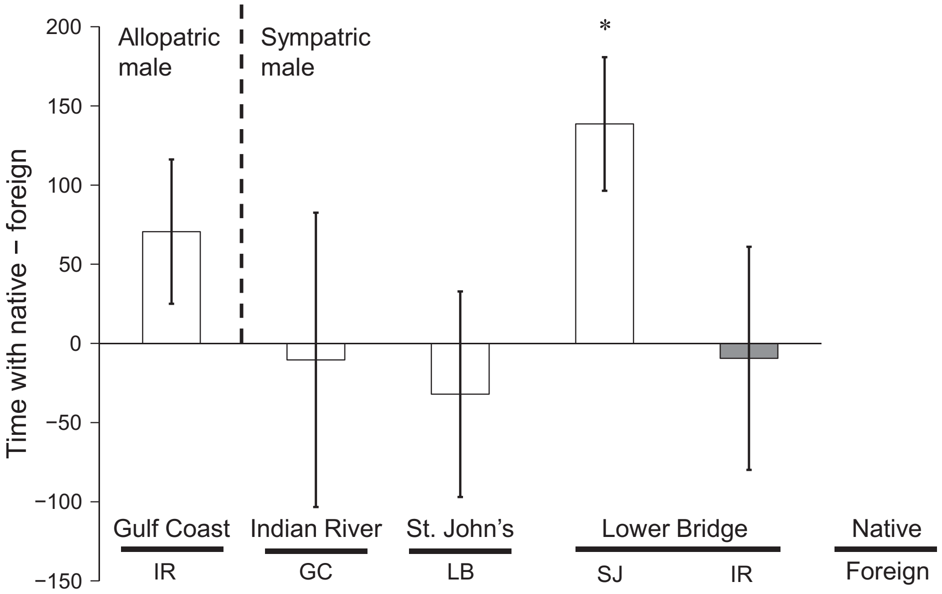

Figure 4: Male *Lucania parva* preference for mates of their native population over mates from foreign populations. Mean (±1 SE) preference for native mates is shown. Native population of focal individual is listed above each line, and foreign mate population is listed below (GC = Gulf Coast; IR = Indian River; SJ = St. John's; LB = Lower Bridge). White bars indicate when both native and foreign *L. parva* populations are from the same salinity, and gray bars indicate populations from different salinities. Gulf Coast *L. parva* are allopatric; all other populations are sympatric with *Lucania goodei*. Significance of preference difference from 0 is indicated by asterisks ($P < .05$).

between focal populations (table A2) or between sympatric or allopatric populations (t-test assuming unequal variance: sympatry vs. allopatry $t_{21.31} = 0.84$, $P = .41$). Males tended to prefer females that were larger, although this effect was not quite significant ($F_{1,35} = 3.08$, $P = .088$; fig. A2). Salinity differences did not enhance male preferences for native females. The observed pattern of male preference was actually the opposite of that predicted: Lower Bridge males showed little preference for native females when foreign females were from a different salinity but tended to prefer native females over foreign females from the same salinity (same vs. different salinity $F_{1,17} = 3.085$, $P = .097$; fig. 4).

The pattern of cascade reinforcement—increased preference for native mates in populations sympatric with *L. goodei*—differed as a function of sex. Sympatric female *L. parva* had stronger preferences for mates from their native populations than allopatric females ($t_{98} = 2.02$, $P = .046$). In contrast, there was no difference between preferences of sympatric and allopatric males (fig. 5). There was also a significant (or marginally significant) interaction between sex and sympatry (absolute preference: $F_{1,98} = 3.50$, $d = 0.59$, $P = .064$; scaled preference: $F_{1,98} = 4.40$, $d = 0.66$, $P = .038$; table A3), suggesting that the relationship between behavioral isolation among populations and sympatry with *L. goodei* varies between males and females in *L. parva*.

The patterns in female mating preferences (as measured with association time) corresponded to actual egg-laying decisions when females had the opportunity to spawn with males. Female egg-laying behavior differed across populations, with females from sympatric populations laying more eggs with males from their native population (fig. 6). In contrast, allopatric females showed no such preference (t-test assuming unequal variance: sympatry vs. allopatry $t_{43.39} = 3.77$, $P = .0004$). Egg-laying preference did not depend on whether males originated from similar or different salinity environments (table A4). This suggests that time association measures of female preference for native males reflect female mating behavior that would lead to behavioral isolation between sympatric populations and foreign populations.

These differences in egg-laying preference between allopatric and sympatric *L. parva* were echoed in a 14-day no-choice assay between a second allopatric population from Texas and the sympatric Indian River population. When paired with a single male for 14 days, the number of eggs produced by females was significantly different between cross types ($F_{3,28} = 4.08$, $P = .016$). Post hoc t-tests indicated that Indian River females produced more eggs with native males ($t_{28} = 3.10$, $P = .004$); the allopatric Texas females showed no such preference ($t_{28} = 1.34$, $P = .19$; fig. A3). This suggests that allopatric females from Texas also lack preference for native males.

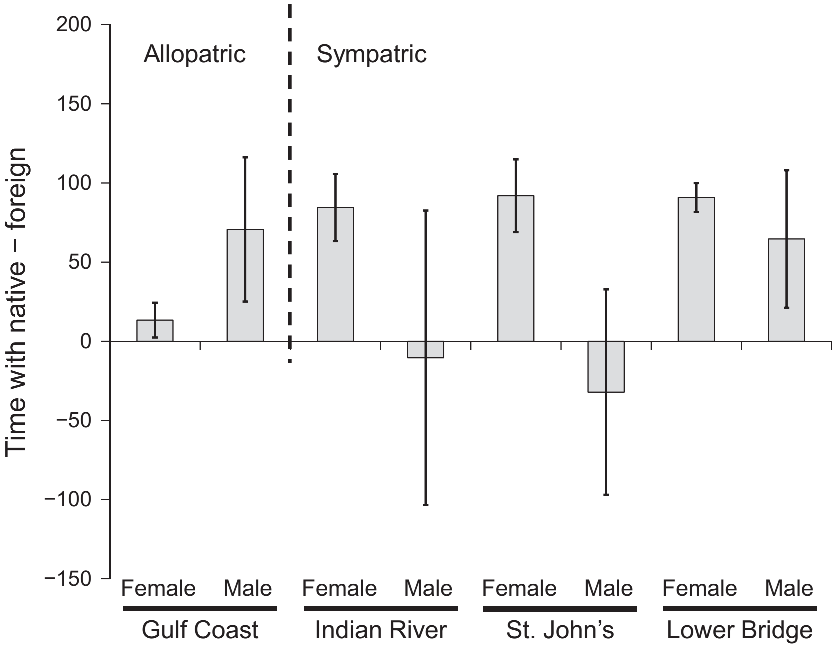

Figure 5: Mate preferences of *Lucania parva* males and females from populations sympatric or allopatric with *Lucania goodei*. Female and male mean (± 1 SE) preference for mates from their native population over foreign mates is shown. The analysis used only population pairs in which both males and females were tested ($n = 5$ pairs). Gulf Coast *L. parva* are allopatric; all other populations are sympatric with *Lucania goodei*.

Male Traits

Across populations of *L. parva*, males differed in traits that may potentially be the targets of mate choice. Both standard length and total anal fin area differed across populations (fig. A4; table A5). Differences in total anal fin area remained among populations even after correcting for the effect of standard length. Sympatric male *L. parva* differed in both morphological measurements (standard length and anal fin area) from male *L. goodei*. Allopatric *L. parva* were more similar to *L. goodei* (fig. A4). Data underlying figures 1–6 and figures A1–A4 are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.ch86c (Kozak et al. 2015).

Discussion

Our results indicate that reinforcement has played a major role in the evolution of behavioral isolation in *Lucania*. Reinforcement has strengthened both male and female preferences between species. Sympatric *Lucania goodei* and *Lucania parva* have strong preferences for conspecific over heterospecific mates. Cascade reinforcement predicts that reinforcement between species creates strong species-specific preferences and that these preferences cause behavioral isolation among populations within species as a correlated response. We find that reinforcement in sympatric *L. parva* leads to behavioral isolation between each sympatric population and all foreign populations. This behavioral isolation among populations of *L. parva* occurs through changes in sympatric female (but not male) preference. These changes in female preference are not associated ecological differences in salinity. Thus, our results suggest that cascade reinforcement is occurring within *L. parva*, providing the first example for this phenomenon in fish.

Our work adds to a growing literature on how the nature of cascade reinforcement may differ between systems. In rock-pool beetles (*Ochthebius urbanelliae*), reinforcement creates isolation between allopatric and sympatric populations, so sympatric populations mate with one another but will not mate with allopatric populations (Porretta and Urbanelli 2012). In *Drosophila subquinaria*, sympatric females reject allopatric males on the basis of epicuticular compounds (Jaenike et al. 2006; Curtis et al. 2013; Giglio and Dyer 2013; Dyer et al. 2014), while in *Drosophila serrata*, allopatric females reject sympatric males (Higgie and Blows 2008). Our results are most similar to those for walking stick insects (Nosil et al. 2003), in which sympatric females preferred native males over males from foreign populations regardless of whether the foreign males were from sympatric or allopatric populations.

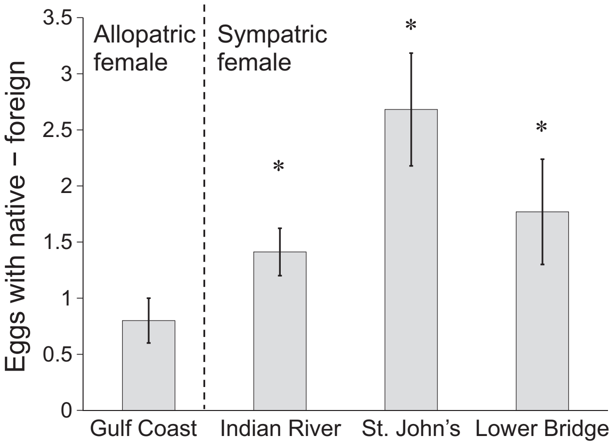

Figure 6: Behavioral isolation between *Lucania parva* populations as a function of spawning. The mean (±1 SE) difference in the number of eggs laid by females in sequential trials with males from their native population versus males from a foreign population is shown. Only females that laid at least one egg with one male were included. Results are indicated for females from Gulf Coast ($n = 10$), Indian River ($n = 17$), St. John's ($n = 22$), and Lower Bridge ($n = 13$). Gulf Coast *L. parva* are allopatric; all other populations are sympatric with *Lucania goodei*. Mean preferences significantly different from 0 are indicated by asterisks ($P < .05$).

Our study is the first to (1) test for cascade reinforcement in male preferences and (2) compare the influence of cascade reinforcement on preferences between the sexes. Surprisingly, our results suggest that cascade reinforcement occurs only in female preference. The lack of male preference among sympatric populations of *L. parva* is unexpected, given that we find evidence for reinforcement of male preference when males are choosing among species.

There are several possible explanations why the pattern of cascade reinforcement is lacking among male *L. parva*. First, males may be prevented from discerning between native and foreign females due to a lack of female population-specific traits that males can use as a basis for preference. The traits (or suites of traits) that males use to differentiate *L. goodei* from *L. parva* may simply not be variable within *L. parva*. Presumably, males and females are using different traits (or suites of traits) to identify conspecifics. Cascade reinforcement could be precluded in males if female traits have lower levels of population variation than male traits in *L. parva*.

A second possibility is that a correlated response does occur in male preference but that there is strong subsequent selection to disentangle within-species from among-species preference. Selection against within-species preference could occur if it is costly for males to reject conspecific females. Sexual selection theory predicts that occasionally incorrectly rejecting conspecific males as mates is unlikely to alter female reproductive success, whereas incorrectly rejecting conspecific females as mates would undoubtedly reduce male reproductive success (Bateman 1948; Trivers 1972). To test these ideas, future work is needed to identify the traits that males and females use to reject heterospecifics and foreign conspecifics.

Asymmetric postzygotic isolation might also generate stronger selection in one sex to avoid heterospecific mating and cause sex-specific cascade reinforcement. Between species, hybrid inviability and sterility effects are often asymmetric, due to mitochondrial-autosomal incompatibilities, sex chromosome evolution, or maternal effects (Bolnick et al. 2008). Asymmetries in postzygotic isolation can cause subsequent asymmetries in behavioral isolation (Jaenike et al. 2006; Yukilevich 2012). In *Lucania*, fertility is reduced by 50% in F_1 hybrid males when female *L. parva* are crossed to male *L. goodei* compared with F_1 males from the reverse cross (female *L. goodei* and male *L. parva*; Fuller 2008*a*). This asymmetry in F_1 fertility may generate strong selection to avoid hybrid matings in female *L. parva* and male *L. goodei*. Although sympatric male and female *L. parva* had similar measured levels of rejection of *L. goodei* in the present study, other data suggest that *L. parva* males have less stringent preferences than females (Berdan and Fuller 2012; G. M. Kozak and R. C. Fuller, unpublished data). For example, in no-choice mating trials between allopatric Blue Springs *L. goodei* and sympatric Indian River *L. parva*, female *L. goodei* and male *L. parva* mate sooner (after 25.63 ± 3.93 days) than female *L. parva* and male *L. goodei* (after 34.5 ± 3.44 days; fig. A5). If these asymmetries in hybrid fitness are driving the direction of cascade reinforcement, we would predict that *L. goodei* should show cascade reinforcement of preference in the opposite direction (in males but not in females). Thus, sex differences in cascade reinforcement in *L. goodei* would be informative for generating testable hypotheses about the way in which reinforcement generates selection on male and female preferences in *Lucania*.

The two main alternative hypotheses to cascade reinforcement are ecological divergent selection and divergent sexual selection. Our data allow us to reject ecological divergent selection as a cause of behavioral isolation in *Lucania*. If ecological differences contributed to behavioral isolation between species, we would expect that freshwater-saltwater pairs would show stronger isolation than freshwater-freshwater pairs. Neither the among-species nor the within-species analysis support this. At the among-species level, the sympatric *L. goodei* and *L. parva* populations occurred in the same salinity yet exhibited strong behavioral isolation. The allopatric populations differed in salinity but lacked behavioral isolation. At the within-species level, behavioral isolation among populations was not a function of differences in population salinity. Previous work has found that behavioral isolation between species remains strong in both freshwater and saltwater mating environments, suggesting that the sensory physiology underlying mate choice is not strongly influenced by salinity (Berdan and Fuller 2012).

At first glance, the lack of an environmental effect on behavioral isolation is surprising given that the signaling environment has a large influence on the signaling dynamics within *L. goodei*. *Lucania goodei* can be found in both clear-water springs and tannin-stained swamps. These different signaling environments have profound effects on color pattern and color vision (Fuller 2002; Fuller et al. 2003, 2004, 2005, 2010). However, in sympatry, *L. parva* and *L. goodei* experience the same lighting environment, and there is no apparent microhabitat partitioning. Similarly, there is no systematic difference in lighting habitat between sympatric and allopatric populations that could contribute to the pattern of cascade reinforcement. However, there may be other unmeasured environmental variables that could conceivably drive divergence in sexual signals and mate preferences, including differences in predators, competitors, or parasites.

Instead of playing a direct role, salinity may contribute indirectly to behavioral isolation in *Lucania* by driving the evolution of hybrid incompatibilities that may provide the basis for reinforcement. We are currently testing this hypothesis. Our preliminary evidence suggests that a number of parallel genetic changes have occurred between freshwater and saltwater populations (particularly in ionoregulatory genes; Kozak et al. 2014) and that hybrid inviability exists between freshwater and saltwater *L. parva* populations (Kozak et al. 2012), although the contribution of genetic drift and isolation by distance to this inviability has not yet been formally tested.

Another alternative to cascade reinforcement is divergent sexual selection. Divergent sexual selection between populations could cause preferences for native males over foreign males; however, this process should occur randomly and not be limited to sympatric populations. Our results indicate that preference for native males was higher in sympatric populations than in allopatric populations. Females from three sympatric populations preferred native males over foreign males, and females from two allopatric populations lacked this preference. Further support for the role played by reinforcement in this system comes from our preliminary analysis of male traits. Male *L. parva* from sympatric populations differed from *L. goodei* in both body length and total anal fin area. In contrast, male *L. parva* from allopatric populations were more similar to *L. goodei*. Both body size and anal fins are important in dominance and courtship signaling in *Lucania* (Fuller 2001; McGhee et al. 2007; McGhee and Travis 2011; Johnson and Fuller 2015). Hence, our findings are consistent with reproductive character displacement driving male trait evolution in sympatric populations.

Surprisingly, sympatric populations varied in the degree to which traits of male *L. parva* differ from those of male *L. goodei*. Differing degrees of divergence are consistent with multiple independent episodes of reinforcement. Atlantic and Gulf contact zones represent separate events of marine *L. parva* invading coastal rivers and encountering upstream populations of *L. goodei*. Genetic data indicate the presence of shared mitochondrial haplotypes between the two species that are unique among drainages, suggesting that each drainage represents a separate hybridization event (K. Murphy and R. C. Fuller, unpublished data). Theory on the repeatability of reinforcement is sparse, and to our knowledge there is no reason to assume that reinforcement acts in the same way in all populations, especially when mate choice can be governed by multiple signals (McPeek and Gavrilets 2006). This may explain why we find that sympatric populations are isolated from other independently derived sympatric populations and not just allopatric populations. More work in other systems is needed to test this idea. In *Lucania*, further work is needed to determine how preferences and traits associated with mate choice (including olfactory, coloration, and courtship behavior) are evolving differently in sympatry and allopatry. We do know that there are significant levels of genetic differentiation between the sympatric populations in this study ($F_{\mathrm{ST}} = 0.11$–13; estimated from transcriptome data; Kozak et al. 2014), suggesting that there may be additional axes of differentiation between populations that could explain the patterns of mate preference we observe.

In summary, we show that reinforcement of mate preferences occurs in both sexes and both species of *Lucania*. Within *L. parva*, asymmetric behavioral isolation is evolving between populations due to the cascading effect of reinforcement on female preferences in sympatric populations. Ecological adaptation to salinity does not appear to contribute directly to the evolution of behavioral isolation within *L. parva*. The female preferences documented here likely reflect actual spawning behavior when males and females can freely interact, as our association-preference data showed the same patterns as our egg-laying assays (see McGhee et al. 2007 for further data on female preference in *Lucania*). Therefore, although the cascade reinforcement effect is limited to female preference, it suggests that female preferences can create isolation between *L. parva* populations. Our work suggests that cascade reinforcement may be a potent force in behavioral isolation, and it deserves to be the subject of future theoretical and empirical work.

Acknowledgments

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