

Original Article

Asymmetric reinforcement in *Lucania* killifish: assessing reproductive isolation when both sexes choose

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Abstract

Reinforcement can occur when maladaptive hybridization in sympatry favors the evolution of conspecific preferences and target traits that promote behavioral isolation (BI). In many systems, enhanced BI is due to increased female preference for conspecifics. In others, BI is driven by male preference, and in other systems both sexes exert preferences. Some of these patterns can be attributed to classic sex-specific costs and benefits of preference. Alternatively, sex differences in conspecific preference can emerge due to asymmetric postzygotic isolation (e.g., hybrid offspring from female A × male B have lower fitness than hybrid offspring from female B × male A), which can lead to asymmetric BI (e.g., female A and male B are less likely to mate than female B and male A). Understanding reinforcement requires understanding how conspecific preferences evolve in sympatry. Yet, estimating conspecific preferences can be difficult when both sexes are choosy. In this study, we use *Lucania* killifish to test the hypothesis that patterns of reinforcement are driven by asymmetric postzygotic isolation between species. If true, we predicted that sympatric female *Lucania goodei* and sympatric male *L. parva* should have lower levels of BI compared with their sympatric counterparts, as they produce hybrid offspring with the highest fitness. To address the problem of measuring BI when both sexes are choosy, we inferred the contribution to BI of each partner using assays where one sex in the mating pair comes from an allopatric population with potentially low preference, whereas the other comes from a sympatric population with high preference. For one hybrid cross direction, we found that both female *L. parva* and male *L. goodei* have high contributions to BI in sympatry. In the other hybrid cross direction, we found that only female *L. goodei* contribute to BI. Sympatric male *L. parva* readily engaged in hybrid spawnings with allopatric *L. goodei* females. These results indicate that both asymmetric postzygotic isolation and the traditional sex-specific costs to preference likely affect the nature of selection on conspecific preferences and target traits.

Key words: reinforcement, behavioral isolation, female mate choice, male mate choice, asymmetric costs to hybridization

Understanding how and why reproductive isolation forms between groups is an outstanding question in evolutionary biology. The initial stages of divergence often occur in allopatry (Mayr 1942; Coyne and Orr 2004; Ridley 2004), but the critical question is what happens to these groups upon secondary contact. Four different

outcomes are possible (Coyne and Orr 2004). First, the levels of reproductive isolation between groups may be sufficient to maintain species boundaries (i.e., good species). Second, levels of reproductive isolation between groups may be insufficient, and groups may collapse into a hybrid swarm. Third, one group may simply outcompete

the other, causing one group to become locally extinct. Finally, groups in secondary contact may hybridize at low levels, and produce maladaptive hybrids. In this scenario, selection against hybrids may increase conspecific preferences within groups, and complete the speciation process in sympatry (Butlin 1987; Noor 1999; Servedio and Noor 2003). This phenomenon, known as reinforcement, was initially met with skepticism, but theoretical and empirical work has since found support for reinforcement.

Early theoretical work considered systems where females acted as choosers and males acted as courters (Liou and Price 1994; Kelly and Noor 1996), and many empirical studies have shown that, indeed, behavioral isolation (BI) among taxa is often due to female mating preferences (Butlin and Ritchie 1991; Rundle and Schluter 1998; Servedio 2007; Dyer et al. 2014). However, other systems have provided good evidence for BI due to male mating preferences (Coyne et al. 1994; Peterson et al. 2005; Servedio 2007; Espinedo et al. 2010; Moran et al. 2017; Moran and Fuller 2018), and others have shown a mix where both females and males have preferences for conspecifics (Gregorio et al. 2012; West and Kodric-Brown 2015; but see Kozak et al. 2009). The question of what determines which sex exerts conspecific preferences and creates BI is unresolved. One possibility is that the classic sex-specific patterns of costs and benefits of choice at the within-species level determine the levels of conspecific preference in males and females. One sex, typically females, often invest more in a given reproductive event and have fewer overall mating attempts than males (Andersson 1994; Shuster and Wade 2003; Clutton-Brock 2007, 2009; Servedio 2007; Kozak et al. 2009). Here, the cost of hybridization may be greater for females than males, leading to high levels of female conspecific preference. Of course, sex ratios, densities of prospective mates, and predation risk can all affect the costs/benefits of choosing and courting (Clutton-Brock and Parker 1992; Kvamemo and Ahnesjö 1996; Shuster and Wade 2003; Jennions and Petrie 2007).

In addition to the effects of classic sexual selection/mating system biology, the nature of postzygotic isolation can also create costs and benefits of choosing and courting that are unique to hybridization between species (Coyne and Orr 2004). Asymmetric postzygotic isolation is common and occurs when hybrids in one direction (e.g., female A × male B) have lower fitness than hybrids in another direction (e.g., female B × male A), and empirical examples of these types of costs have been documented in insects (Shapiro 2006; Hochkirch and Lemke 2011; Sánchez-Guillén et al. 2012; Yukilevich 2012), fish (Crow et al. 2007; Van Der Sluijs et al. 2008; Martin and Mendelson 2013), mammals (Smadja and Ganem 2005; Beysard et al. 2015; Shipley et al. 2016), amphibians (Pfennig and Simovich 2002; Arntzen et al. 2009), and plants (Tiffin et al. 2001; Ramsey et al. 2003; Coughlan and Willis 2018). In these scenarios, the expectation is that BI will be concordant with the direction of postzygotic isolation (i.e., females of species A and males of species B will be less likely to engage in hybrid matings than females of species B and males of species A). Theoretically, this variation in hybridization costs may affect the patterns of reproductive character displacement and, ultimately, the outcomes of reinforcing selection (Veen et al. 2001; Pfennig and Simovich 2002; Clutton-Brock 2007; Yukilevich 2012).

The *Lucania* system is excellent for investigating how variation in hybridization costs may affect patterns of reproductive character displacement, and reinforcement, for several reasons: The *Lucania* system contains 2 closely-related sister species (Duggins et al. 1983; Whitehead 2010)—the bluefin killifish (*L. goodei*) and the rainwater killifish (*L. parva*). *L. goodei* and *L. parva* can be found in sympatry

and allopatry across Florida, with additional populations of allopatric *L. parva* across the Atlantic coast and the Gulf of Mexico. Previous studies suggest that these sister species diverged in allopatry and, in some populations, came back into secondary contact approximately 2 million years ago (Ghedotti and Davis 2017). Behavioral and genetic data also suggest that conspecific mate preference is stronger in sympatry compared with allopatry for both *L. goodei* and *L. parva*, a pattern consistent with reproductive character displacement and reinforcement (Fuller et al. 2007; Berdan and Fuller 2012; Gregorio et al. 2012; Kozak et al. 2015). Therefore, there is good support for reinforcement and a pattern of reproductive character displacement in this system.

Second, there is variation in hybridization costs due to sex and species identity in the *Lucania* system that may affect the outcome of reinforcement. Previous studies have documented asymmetric fitness costs to hybridization due to species identity and cross direction, where male F1 hybrids, produced from male *L. goodei* and female *L. parva* parents, suffer a significant reduction in fitness compared with hybrids formed from female *L. goodei* and male *L. parva* (Fuller 2008). Backcrosses into *L. goodei* also suffer reduced fitness whereas backcrosses into *L. parva* do not. Reinforcement via this process predicts that male *L. goodei* and female *L. parva* should have increased levels of conspecific mate preference compared with female *L. goodei* and male *L. parva* in sympatry. On the contrary, the *Lucania* system follows traditional sex roles and females energetically invest more into reproductive events than males, suggesting that females of both species may have increased levels of conspecific preference in sympatry compared with their male counterparts. Evidence supporting these predictions is mixed (Berdan and Fuller 2012; Kozak et al. 2015; St. John and Fuller 2019), and no clear connection between variation in hybridization costs and reinforcement has been documented in this system.

In this study, we used no-choice assays to investigate the roles of sex and cross direction on the patterns of conspecific mate preference in the *Lucania* system (hereafter referred to as BI). We hypothesize that hybridization costs associated with the asymmetry in the genetic incompatibility (i.e., cross direction) may cause concordant patterns of asymmetry in BI, and predict that *L. goodei* females and *L. parva* males from sympatric populations should have lower levels of conspecific preference than *L. parva* females and *L. goodei* males from the same populations. Alternatively, the sex-specific costs of reproduction and hybridization predict that conspecific preference may be higher for females of both species in sympatry compared with their male counterparts. We found that male *L. parva* from sympatry contributed less to BI than all other sympatric groups, supporting the hypothesis that the asymmetry in the genetic incompatibility alters the nature of reproductive character displacement. However, we found that female *L. goodei*—who we expected to follow the same pattern as male *L. parva*—had consistently high levels of BI. We conclude that the patterns of BI in the killifish system cannot solely be explained by the asymmetric costs to hybridization that genetic incompatibilities and cross direction produce. Instead, we suggest alternative factors that may be affecting the patterns of BI observed in this system.

Materials and Methods

Collection and care

During the summers of 2015 and 2016, we collected 4 types of populations for this study: 1) a sympatric population of *L. goodei* from Salt Springs (Marion County, FL; St. John's river drainage), 2)

sympatric populations of *L. parva* from California Creek (Dixie County, FL; Suwannee river drainage) and Salt Springs (Marion County, FL; St. John's river drainage), 3) an allopatric population of *L. goodei* from Blue Springs (Gilchrist County, FL; Santa Fe river drainage), and 4) an allopatric population of *L. parva* from Lake Pontchartrain (St. Tammany County, LA) (for a complete list of sympatric and allopatric populations of *Lucania* across Florida, see Fuller and Noa 2008). We used dip nets and seines to collect males and females from each of these populations. Using coolers, we transported the fish back to the University of Illinois Urbana-Champaign where they resided for the duration of the study period. At the University of Illinois Urbana-Champaign, fish were kept in large cattle tanks in an outdoor greenhouse. Fish were exposed to natural light cycles and were fed a diet of brine shrimp and blood worms daily.

Egg production as a metric of male and female preference

We used the total number of eggs produced by mate pairs in a no-choice assay as a proxy for mate preference for each of the 4 population types. Typically, male killifish hold small territories around vegetation, where females visit them. During these visits, a female may assess a male and vice versa. If a male wish to mate with a female, the male will begin courting her by shaking his head and swimming around her. During this time, a female may: 1) continue to assess the male, 2) decide that she is willing to mate with him, or 3) decide that she is uninterested and swim away. If a female decides to mate with a male, they swim side by side and deposit eggs on vegetation in the male's territory. Killifish only deposit 1–2 eggs per spawning bout—and females do not deposit eggs in isolation or without a male spawning partner—indicating that number of eggs produced from a spawning pair is a good proxy for the number of spawning bouts, and thus a good measure of preference for both individuals.

Number or frequency of spawning/mating bouts is a common metric of preference across many different systems (Hoikkala and Aspi 1993; Coyne et al. 2005; Schöfl et al. 2011; Dougherty and Shuker 2014), and our previous work has explicitly investigated the ability of egg production—along with other behavioral metrics—to detect conspecific preference in *Lucania*. Although measurement of association time and the number of courting bouts reliably detected preference for male *L. goodei*, they were not good measures of preference for female *L. goodei*. Instead, we found that number of eggs produced in no choice assays could reliably detect conspecific mate preference for both male and female *L. goodei*, which is why we use this measurement here (St. John and Fuller 2019).

One challenging aspect of using no-choice assays and egg production as a proxy of mate preference is that it can be difficult to parse out the relative contributions of the female and male to BI. Previous studies investigating reinforcement have traditionally compared estimates of BI using conspecific and heterospecific pairs from sympatry and allopatry (Figure 1). These population pairs are obviously required to compare the levels of BI for sympatric versus allopatric populations. However, the use of traditional sympatric and allopatric population pairs does not inform as to the contributions of each species and sex to BI. For example, a pairing between a female of species A and a male of species B may fail to mate. The traditional assumption is that a failure to mate is a property of female mating preference and male traits. However, in systems where both sexes choose, both sexes can act as choosers and both sexes possess

target traits that the other sex can assess. To address this, we repeatedly estimated preferences for each individual by producing conspecific and heterospecific pairs with individuals from both sympatry and allopatry (4 mates per individual). Repeated measures of an individual's mate preference typically provide a more precise estimate of said preference (Wagner 1998; Dougherty and Shuker 2015). Furthermore, for our sympatric individuals, measuring their mate preference using mates from allopatry—who presumably have weaker or no conspecific mate preference—allows us to parse out the contributions of a given individual to a mating pair.

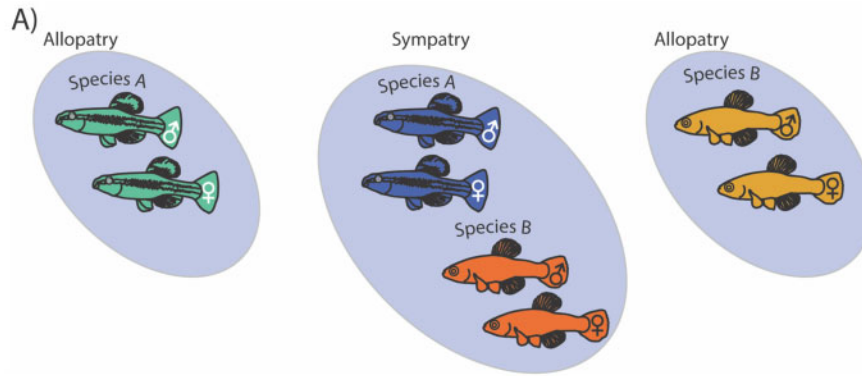
Experimental design

In total, we measured mate preference for 20 allopatric *L. goodei* individuals (10 males and 10 females from Blue Springs), 20 sympatric *L. goodei* individuals (10 males and 10 females from Salt Springs), 14 allopatric *L. parva* individuals (6 males and 8 females from Lake Pontchartrain, LA), and 26 sympatric *L. parva* individuals (11 males and 9 females from California Creek; 3 males and 3 females from Salt Springs). To measure preference for all 80 individuals, we set up 40 aquaria comprised 10 blocks of 4 tanks. Four males (a sympatric *L. goodei* male, an allopatric *L. goodei* male, an allopatric *L. parva* male, and a sympatric *L. parva* male) and 4 females (a sympatric *L. goodei* female, an allopatric *L. goodei* female, an allopatric *L. parva* female, and a sympatric *L. parva* female) were randomly assigned to each block. Within each block, we paired each male and female fish over the course of 5 weeks in July and August 2016. At the end of the study period, each block produced data for 16 unique male–female pairings (see Figure 2 for all pair types). In total, 10 replicates produced data for 160 unique pairings.

Administration of assays

Immediately preceding the start of the study, we randomly assigned females to 1 of the 4 38-L tanks in their block. Females remained in their individual tanks for the entirety of the study, whereas males were moved between female tanks (but stayed in their assigned block). Each individual experienced 4 no-choice assays, which each lasted for 10 days. Assays began when a male was placed into a female tank along with 2 top mops (yarn attached to a Styrofoam ball) and 2 bottom mops (yarn attached to PVC pipe) that served as spawning substrate. The first 3 days of the no-choice assay were used as acclimation time for the mating pair. Any eggs collected during this time were disregarded. During the remaining 7 days, we collected and counted eggs from each mating pair and used the total number of eggs produced during this time as a proxy for preference. After egg collection on the 7th day, we removed males from their assigned tanks, randomly paired them with another female in their block, and repeated the process. We also followed this procedure for Weeks 3 and 4 so that all males were paired for 1 week (not including the acclimation time) with all females.

One caveat for this design is that we have a smaller sample size of *L. parva* from Lake Pontchartrain (6 males, 8 females). We had also hoped to use sympatric *L. goodei* and *L. parva* from the same location, but only had 3 males and 3 females of Salt Springs *L. parva*. To bolster the sample size and ensure that all animals experienced the same number of mates across replicate groups, we supplemented sympatric *L. parva* from California Creek (a separate sympatric site) wherever there was a missing *L. parva*. For blocks missing a sympatric *L. parva* from the Salt Springs population, we simply used animals from California Creek. For blocks missing an



B) Female Behavioral Isolation	Female Geography	Female Species	Male Geography	Male Species	Pair Type
BI: Sympatric ♀ A x sympatric ♂ B	Sympatric	A	Sympatric	A	Conspecific
	Sympatric	A	Sympatric	B	Heterospecific
BI: Sympatric ♀ A x Allopatric ♂ B	Sympatric	A	Sympatric	A	Conspecific
	Sympatric	A	Allopatric	B	Heterospecific
BI: Allopatric ♀ A x Sympatric ♂ B	Allopatric	A	Allopatric	A	Conspecific
	Allopatric	A	Sympatric	B	Heterospecific
BI: Allopatric ♀ A x Allopatric ♂ B	Allopatric	A	Allopatric	A	Conspecific
	Allopatric	A	Allopatric	B	Heterospecific
BI: Sympatric ♀ B x Sympatric ♂ A	Sympatric	B	Sympatric	A	Heterospecific
	Sympatric	B	Sympatric	B	Conspecific
BI: Sympatric ♀ B x Allopatric ♂ A	Sympatric	B	Allopatric	A	Heterospecific
	Sympatric	B	Sympatric	B	Conspecific
BI: Allopatric ♀ B x Sympatric ♂ A	Allopatric	B	Sympatric	A	Heterospecific
	Allopatric	B	Allopatric	B	Conspecific
BI: Allopatric ♀ B x Allopatric ♂ A	Allopatric	B	Allopatric	A	Heterospecific
	Allopatric	B	Allopatric	B	Conspecific

C) Male Behavioral Isolation	Female Geography	Female Species	Male Geography	Male Species	Pair Type
BI: Sympatric ♂ A x sympatric ♀ B	Sympatric	A	Sympatric	A	Conspecific
	Sympatric	B	Sympatric	A	Heterospecific
BI: Sympatric ♂ A x Allopatric ♀ B	Sympatric	A	Sympatric	A	Conspecific
	Allopatric	B	Sympatric	A	Heterospecific
BI: Allopatric ♂ A x Sympatric ♀ B	Allopatric	A	Allopatric	A	Conspecific
	Sympatric	B	Allopatric	A	Heterospecific
BI: Allopatric ♂ A x Allopatric ♀ B	Allopatric	A	Allopatric	A	Conspecific
	Allopatric	B	Allopatric	A	Heterospecific
BI: Sympatric ♂ B x Sympatric ♀ A	Sympatric	A	Sympatric	B	Heterospecific
	Sympatric	B	Sympatric	B	Conspecific
BI: Sympatric ♂ B x Allopatric ♀ A	Allopatric	A	Sympatric	B	Heterospecific
	Sympatric	B	Sympatric	B	Conspecific
BI: Allopatric ♂ B x Sympatric ♀ A	Sympatric	A	Allopatric	B	Heterospecific
	Allopatric	B	Allopatric	B	Conspecific
BI: Allopatric ♂ B x Allopatric ♀ A	Allopatric	A	Allopatric	B	Heterospecific
	Allopatric	B	Allopatric	B	Conspecific

Figure 1. (A) Species A and B and their geographic relationships. (B) Crosses used to measure BI for females as a function of sympatry and allopatry. (C) Crosses used to measure BI for males as a function of sympatry and allopatry. Traditional crosses used to diagnose RCD in white. Nontraditional crosses used to diagnose the roles of species and sex are shown in gray fill.

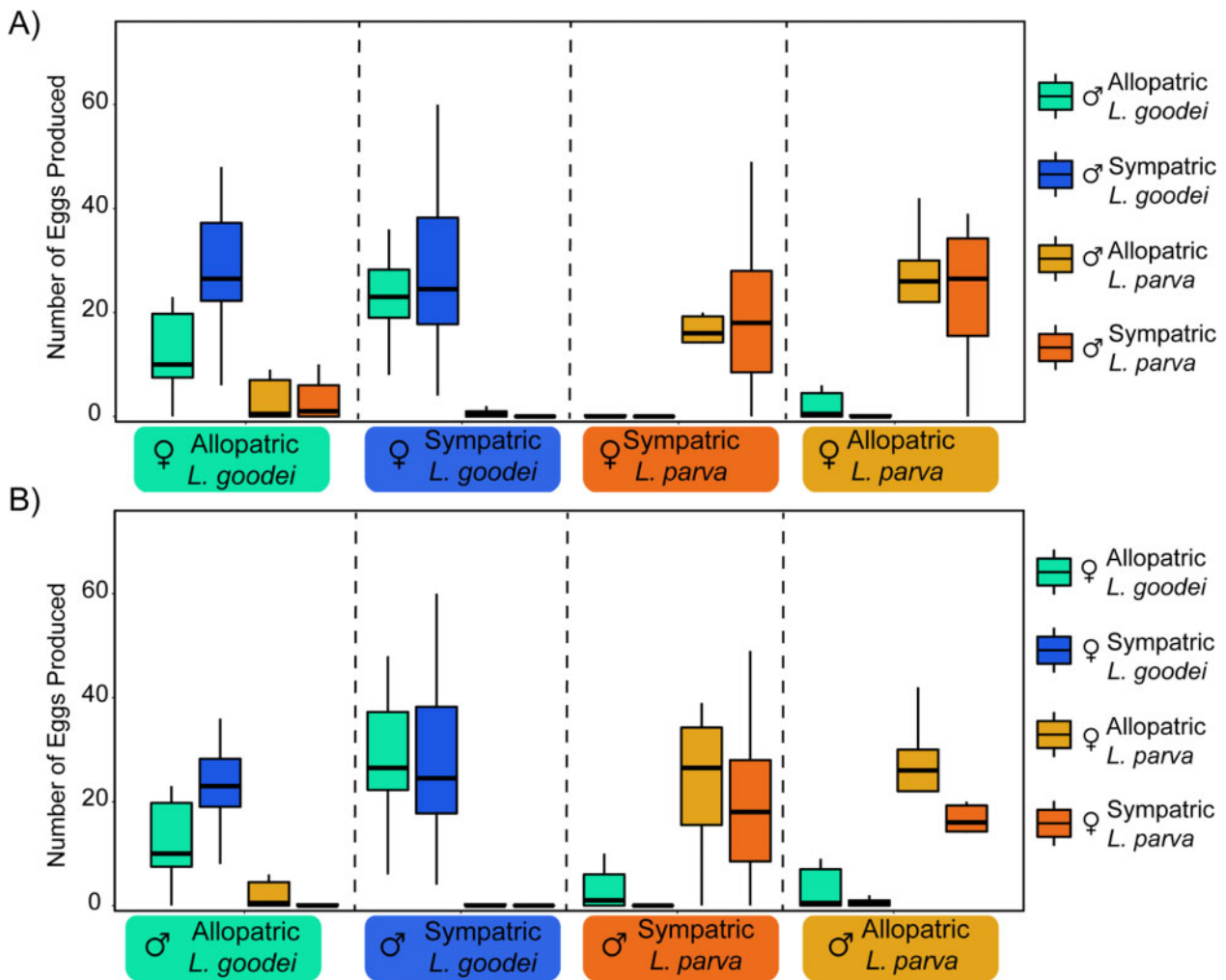


Figure 2. Number of eggs produced by 16 unique mate pair types. (A) The 16 pairs from the female perspective. (B) The 16 pairs from the male perspective. The x-axis shows species and geographic designations, whereas boxplot colors indicate species and geographic designations of the mate.

allopatric *L. parva* from Lake Pontchartrain, we used *L. parva* from California Creek as a “filler animal.” This ensured that all of the animals in the block could spawn for the same number of days. Hence, all blocks had male and female *L. parva* from a sympatric population, but 2 blocks lacked an allopatric *L. parva* female and 4 lacked an allopatric *L. parva* male. Additionally, one individual expired mid-assay and was thus removed from the dataset.

Statistics

Generalized linear mixed models

We used a GLMM to determine if the different combination of species and geography affected the total number of eggs a pair produced. First, we investigated whether block or week should be included in our overall model. We used a GLM with a negative binomial distribution to determine if either of these factors affected the total number of eggs produced by pairs. We found no effect of week ($\chi^2 = 4.95$, $df = 3$, $P = 0.18$) or block ($\chi^2 = 2.94$, $df = 9$, $P = 0.97$) on the total number of eggs produced by pairs, and therefore did not include them in our final model. As part of our experimental design, we purposefully randomized the order in which females were exposed to males in an effort to reduce order effects. We used a GLM with a negative binomial distribution to investigate whether

the order of exposure to conspecific males or the order of exposure to native conspecific males affected the total number of eggs produced by subsequent pairs, but found no effect of either factor (conspecific exposure: $LR \chi^2 = 0.14$, $df = 1$, $P = 0.71$; native conspecific exposure: $LR \chi^2 = 0.10$, $df = 1$, $P = 0.75$). Next, we used *t*-tests to investigate whether the supplemental individuals added to blocks with missing mates were statistically different from their original groups. We found that neither the additional “filler” females ($t = -0.053$, $df = 8.50$, $P = 0.96$) or “filler” males ($t = -0.78$, $df = 17.57$, $P = 0.44$) were statistically different from their original groups and therefore included them with their appropriate groups for the overall model. For our final model, we used a GLMM with a negative binomial distribution and included the total number of eggs produced by a pair as the response variable in the model. Female species, male species, female geography, male geography, and the interactions between these variables were all included as fixed effects. We also included female and male ID as separate random effects (Table 1).

Measuring BI

To make direct comparisons between groups, we used a standardized formula to quantify BI. We used Stalker’s isolation index

Table 1. Results of GLMM to determine how species designation, geography, and their interaction affect the total number of eggs produced by a pair

Response	Predictors	χ^2	df	P-value
Number of eggs produced by a pair	♀ Species	4.736	1	0.03
	♂ Species	1.463	1	0.226
	♀Geography	1.565	1	0.211
	♂Geography	1.856	1	0.173
	♀Species: ♂Species	7.85	1	0.005
	♀Species: ♀Geography	13	1	0.0003
	♂Species: ♀Geography	10.239	1	0.001
	♀Species: ♂Geography	11.428	1	0.001
	♂Species: ♂Geography	1.315	1	0.252
	♀Geography: ♂Geography	0.779	1	0.377
	♀Species: ♂Species: ♀Geography	14.117	1	0.0002
	♀Species: ♂Species: ♂Geography	8.012	1	0.005
	♀Species: ♀Geography: ♂Geography	4.753	1	0.029
	♂Species: ♀Geography: ♂Geography	0.004	1	0.95
	♀Species: ♂Species: ♀Geography: ♂Geography	1.378	1	0.24

Significant predictors are indicated in bold.

(1942) with total number of eggs produced with a mate as a proxy for mate preference:

$$\frac{(\text{Total Eggs Produced with Conspecific Mate}) - (\text{Total Eggs Produced with Heterospecific Mate})}{(\text{Total Eggs Produced with Conspecific Mate}) + (\text{Total Eggs Produced with Heterospecific Mate})}$$

Stalker's isolation index ranges from -1 to $+1$, with negative values representing heterospecific preference, positive values representing conspecific preference, and these values represent a linear relationship between mate preference and behavioral/reproductive isolation (Stalker 1942; Sobel and Chen 2014). Using this formula, we measured BI in 2 different ways: First, by comparing the number of eggs a group (i.e., male sympatric *L. goodei*, female allopatric *L. parva*, etc.) produced with conspecific mates (either *L. goodei* or *L. parva*) from their home population versus the number of eggs a group produced with heterospecific mates from a population of the same geography (i.e., sympatry or allopatry). For example, we calculated BI for sympatric *L. goodei* females by considering the number of eggs females laid with sympatric *L. goodei* males and sympatric *L. parva* males. For allopatric *L. goodei* females, we considered the number of eggs produced when paired with allopatric *L. goodei* males and allopatric *L. parva* males. This allowed us to calculate BI for each sex of each species in both ♀ sympatric: ♂ sympatric crosses and ♀ allopatric: ♂ allopatric crosses (8 measures total). We refer to this as the traditional pairings (Figure 1; Table 2).

Second, we compared the number of eggs a group produced with conspecific mates from their home population versus the number of eggs they produced with heterospecific mates from a population of the opposite geography (e.g., if the focal individual is from sympatry, we paired them with a heterospecific from allopatry). For example, we calculated BI for sympatric *L. goodei* females by considering the number of eggs females laid with sympatric *L. goodei* males and allopatric *L. parva* males. For allopatric *L. goodei* females, we considered the number of eggs produced when paired with allopatric *L. goodei* males and sympatric *L. parva* males. This allowed us to calculate BI for each sex of each species in both ♀ sympatric: ♂ allopatric crosses and ♀ allopatric: ♂ sympatric crosses (8 measures total). We refer to these additional pairings as nontraditional pairings (Figure 1; Table 2).

Although all 16 types of pairs are represented in the dataset, they were not present in equal numbers. The unequal numbers prevented us

from calculating BI values for each individual. Instead, we used a bootstrap resampling method to calculate BI and 95% confidence intervals. We calculated BI for each group for 10,000 replicates. BI values were considered significant if 95% confidence intervals did not overlap with zero (Table 2). All analyses were performed in R (version 3.5.1).

Results

Traditional measures of conspecific preference reveal a pattern of reproductive character displacement in the *Lucania* system

Figure 2 shows the number of eggs produced for each cross-type from 1) the female perspective (Figure 2A), and 2) the male perspective (Figure 2B). Table 2 shows the patterns of BI that emerge from these crosses. There is a clear pattern of reproductive character displacement that is consistent with reinforcement when considering traditional measures of conspecific preference. Conspecific crosses produced many more eggs than did heterospecific crosses (*post hoc* pairwise Wilcoxon rank-sum test with a Bonferroni correction; $P = 7.4 \times 10^{-11}$) and this pattern was heightened as a function of sympatry versus allopatry. Heterospecific crosses involving animals from sympatric populations produced few (if any) eggs in comparison to heterospecific crosses from allopatric populations (Figure 2, *post hoc* pairwise Wilcoxon rank-sum test with a Bonferroni correction; $P = 0.0055$). Estimates of BI from traditional assays show that sympatric groups have strong, significant, conspecific preferences because their estimates are large positive values, that do not overlap with zero (which represents no mate preference; Table 2). Conversely, allopatric groups exhibited much lower estimates of BI that did overlap with zero indicating that, in general, they had weaker or nonexistent conspecific mate preferences (Table 2). However, there is a trend for slightly higher BI for crosses between male *L. goodei* and female *L. parva* in comparison to crosses between male *L. parva* and female *L. goodei*.

Nontraditional crosses show that male *L. parva* contribute less to BI than all other sympatric groups

Nontraditional crosses, involving a combination of animals from sympatric and allopatric populations, allow us to determine which

Table 2. Estimated levels of BI for traditional and nontraditional crosses for *L. goodei* ♀ × *L. parva* ♂ and *L. parva* ♀ × *L. goodei* ♂

<i>L. goodei</i> ♀ × <i>L. parva</i> ♂	Female BI (CL)	Male BI (CL)
Sympatric <i>L. goodei</i> ♀ × sympatric <i>L. parva</i> ♂	0.98 (0.93,1)	0.97 (0.9,1)
Sympatric <i>L. goodei</i> ♀ × allopatric <i>L. parva</i> ♂	0.95 (0.89,1)	0.95 (0.82, 1)
Allopatric <i>L. goodei</i> ♀ × sympatric <i>L. parva</i> ♂	0.41 (−0.4, 0.86)	0.52 (0.18, 0.89)
Allopatric <i>L. goodei</i> ♀ × allopatric <i>L. parva</i> ♂	0.33 (−0.24,1)	0.54 (−0.12,1)
<i>L. parva</i> ♀ × <i>L. goodei</i> ♂	Female BI (CL)	Male BI (CL)
Sympatric <i>L. parva</i> ♀ × sympatric <i>L. goodei</i> ♂	0.97 (0.9, 1)	0.98 (0.93, 1)
Sympatric <i>L. parva</i> ♀ × allopatric <i>L. goodei</i> ♂	0.95 (0.88, 1)	0.94 (0.83, 1)
Allopatric <i>L. parva</i> ♀ × sympatric <i>L. goodei</i> ♂	0.97 (0.88, 1)	0.97 (0.92,1)
Allopatric <i>L. parva</i> ♀ × allopatric <i>L. goodei</i> ♂	0.68 (0.15, 0.99)	0.52 (−0.00063, 0.97)

BI estimates were calculated using stalker's isolation index, and confidence intervals were calculated using bootstrapping methods (10,000 iterations). Traditional crosses are shown in white. Nontraditional crosses are shown in gray.

partner has larger effects on BI. We first concentrate on crosses between *L. parva* females and *L. goodei* males, which produce offspring with reduced hybrid fitness and are predicted to have high BI. The inclusion of either a sympatric *L. parva* female or a sympatric *L. goodei* male creates high BI in this cross direction, and both non-traditional cross types produce high BI (Table 2). The implication is that both *L. goodei* males and *L. parva* females differ in preference/target traits between allopatric and sympatric populations such that both sexes contribute to BI.

Crosses in the opposite direction, *L. goodei* females crossed with *L. parva* males, produce hybrids with higher fitness and are predicted to have lower BI in comparison to the reciprocal hybrid cross. Here, estimates of BI for crosses involving allopatric *L. goodei* females do not differ from zero, regardless of the population of origin of *L. parva*, but estimates of BI for crosses involving sympatric *L. goodei* females are always significantly different from zero, indicating strong conspecific preference (Table 2). Conversely, *L. parva* males from sympatric populations appear to be willing to mate with *L. goodei* females. Specifically, male sympatric *L. parva* produced significantly more eggs when their female *L. goodei* mate was from an allopatric population compared with a sympatric population (*post hoc* pairwise Wilcoxon rank-sum tests with a Bonferroni correction; $P = 0.0072$), and BI estimates fell from 0.97 (CI 0.90–1) to 0.52 (CI 0.18–0.89) when *L. goodei* females were from allopatry compared with sympatry. The interpretation is that sympatric *L. parva* males will more readily engage in hybrid mating events than will *L. goodei* males.

Finally, our GLMM shows that the interaction between female species, male species, and female geography ($\chi^2 = 14.117$, $df = 1$, $P = 0.0002$) and the interaction between female species, male species, and male geography ($\chi^2 = 8.012$, $df = 1$, $P = 0.005$) were both significant predictors for the number of eggs produced by a pair. This result not only suggests that there is variation in the total number of eggs produced by conspecific versus heterospecific pairings (as predicted by reinforcement), but that the number of eggs produced from a conspecific or heterospecific pairing may also depend on whether a mate is from sympatry or allopatry (i.e., whether their preference was measured in a traditional or nontraditional assay).

Discussion

In this study, we aimed to test whether the costs of hybridization associated with cross direction and sex affect the pattern of conspecific mate preference in the *Lucania* system. We made 2 predictions:

First, the hypothesis that asymmetric genetic incompatibilities lead to asymmetric BI predicts that *L. goodei* females and *L. parva* males from sympatric populations should have lower levels of conspecific preference than *L. parva* females and *L. goodei* males from the same populations. Second, the hypothesis that traditional costs/benefits of mate preference in traditional mating systems also affect the costs/benefits of conspecific preference predicts that females of both species should have high BI relative to males in sympatry. Ultimately, we found that sympatric male and female *L. goodei*, and sympatric female *L. parva* had high contributions to BI. Regardless of the geographic identity of their conspecific or heterospecific mate partners, sympatric male and female *L. goodei*, and sympatric female *L. parva* did not engage in hybrid matings at high levels (Table 2; Figure 2). However, we found that sympatric male *L. parva* produced more eggs with heterospecific partners when said partner was from an allopatric population (Table 2; Figure 2)—suggesting that they have weaker conspecific mate preferences and lower contributions to BI than all other sympatric groups. Taken together, these results suggest that neither hypothesis alone fully explains the pattern of BI observed in the *Lucania* system. Instead, we suggest that costs associated with both sex and cross direction may be acting together to influence the species- and sex-specific patterns in reproductive character displacement.

Asymmetric postzygotic isolation does not solely explain the pattern of BI in *Lucania*

The asymmetric postzygotic isolation between *Lucania* species is well documented (Fuller 2008) and predicts that male *L. goodei* and female *L. parva* from sympatric populations should have high conspecific preference. We found strong evidence supporting this prediction. Both male *L. goodei* and female *L. parva* from sympatric populations abstained from hybrid matings regardless of whether they were paired with sympatric or allopatric heterospecifics (Table 2). Likewise, the asymmetry in postzygotic isolation also predicts that female *L. goodei* and male *L. parva* from sympatric populations should have lower levels of BI. However, we found that this prediction was not completely supported. We found that male *L. parva* from sympatric populations readily engaged in hybrid matings when they were paired with allopatric female *L. goodei* in our non-traditional assays (Table 2). This finding supports the predictions of reinforcement and indicates that previously strong estimates of conspecific preference for sympatric male *L. parva* (Fuller et al. 2007; Gregorio et al. 2012; Kozak et al. 2015) were at least partially due

to the mate preferences or traits of the heterospecific mate. However, we also found that female *L. goodei* from sympatric populations exhibited high levels of conspecific preference regardless of whether they were paired with sympatric or allopatric heterospecific males—a finding that does not support our predictions.

Alternative explanations for patterns of BI

If female *L. goodei* produce F1 hybrids with relatively high fitness, then why do they continually exhibit high levels of conspecific preference? One possibility is that selection has favored increased conspecific preference in female *L. goodei* due to the low fitness of their F2 backcrosses. Fuller (2008) found that F1 hybrids with an *L. goodei* mother exhibited no difference in fitness compared with purebred crosses, but F2 generations had extremely low viability when backcrossed into *L. goodei*. This was not the case for *L. parva* hybrids. Instead, F1 hybrids with *L. parva* mothers exhibit significantly lower fitness than purebred F1s, but when backcrossed into *L. parva* the F2 generation exhibited fitness levels on par with purebreds (Fuller 2008). Hence, even though crosses between *L. goodei* females and *L. parva* males create F1 offspring with high viability and high fertility, crosses between *L. goodei* females and F1 hybrid males reduces offspring survival. One caveat for this explanation is that this hypothesis needs a theoretical model to determine whether selection against backcrossed offspring could alter levels of conspecific preference—especially considering that these F1 animals are rare in nature (Hubbs 1955), which would diminish the strength of selection on conspecific preference.

A second possibility is that female *L. goodei* incur additional costs to hybridization that are not experienced by male *L. parva*. For example, females generally invest more in reproduction (i.e., production of eggs, fewer reproductive events) than their male counterparts (Clutton-Brock and Parker 1992; Hayward and Gillooly 2011; Lipshutz 2018). Previous studies have shown that female *L. goodei* have stronger conspecific mate preference than their male counterparts and even exhibit preferences consistent with cascade reinforcement (St. John and Fuller 2019). Additional studies using *Drosophila* also indicate that rapid evolution of female mate preference via reinforcement may even curtail the evolution of male preference (Yukilevich and Peterson 2019). It is possible that reinforcement acted to increase conspecific mate preference for male *L. goodei* and female *L. parva* due to poor hybrid fitness, and also acted to increase conspecific mate preference for female *L. goodei* due to the costs associated with egg production. The data here support both scenarios.

It is also possible that the patterns of BI and conspecific preference observed in the *Lucania* system are not the result of reinforcement or selection against hybrids. Instead, these patterns could be 1) the incidental by-product of differences in selection or gene flow between populations (Coyne and Orr 2004; Cooley 2007), 2) the result of differences in sexual selection across populations (Langerhans and Riesch 2013), or 3) due to selection on a magic trait (Servodio et al. 2011). There is some support for these possibilities in our data. For example, Figure 2A shows that allopatric *L. goodei* females produced more eggs with sympatric *L. goodei* males than with *L. goodei* males from their home population. This could suggest that sexual selection in sympatry has shifted male *L. goodei* target traits, subsequently making them more attractive. However, both traditional and nontraditional estimates of BI measurements indicate that *L. goodei* females from allopatry ultimately exhibit non-significant conspecific preferences, suggesting that future work is needed to confirm or rule out this possibility.

Differences in natural selection across populations may have also incidentally shifted mating traits and preferences in the *Lucania* system. For example, previous studies have documented differences in lighting environment across killifish populations in Florida and have connected this variation to differences in male coloration (Fuller 2002; Fuller et al. 2010). It could be that the differences in conspecific preference and BI observed in this study are due to variation in male traits because of natural selection. However, the role of female mate choice in establishing the population patterns in lighting environment and male coloration is unclear (Fuller and Noa 2010; Mitchem et al. 2018). Finally, the effects of variation in hybridization costs on BI may vary with time since initial secondary contact. The process of reinforcement is expected to increase BI between groups over time, however, once groups approach complete isolation reinforcing selection becomes weaker. Furthermore, as time passes other forces, such as drift or natural selection may erase the patterns of BI produced through variation in hybridization costs.

Experimental design can affect measures of BI

Our novel experimental design also shows that high levels of BI between groups can be due to the behavior/target traits of one or both sexes. We found low levels of BI when using animals from allopatric populations. This pattern is consistent with the predictions of reinforcement and was wholly expected. However, for most crosses, BI increased dramatically when allopatric animals were paired with sympatric heterospecifics (Table 2). The increase in BI can be attributed to the preference/target traits of the sympatric animal. Reproductive character displacement (i.e., increased BI in sympatry) can be diagnosed by comparing BI for sympatric and allopatric animals. However, assessing the relative effect of each sex of each species requires measuring BI in all combinations of species, sex, and geography (i.e., allopatry vs. sympatry).

One caveat for our chosen design is that we used no-choice assays, which have some clear pros and cons with regard to documenting mate preference (Wagner 1998; Dougherty and Shuker 2015; Ryan and Taylor 2015). The advantage of no-choice assay is that they directly measure mating, which is the ultimate behavior of interest. However, there are clear disadvantages with regard to identifying the precise traits that create BI. In the case of the *Lucania* system, there are differences in male color patterns between spring and swamp populations in *L. goodei*, and there are differences in anal fin size between sympatric and allopatric populations of *L. parva* (Kozak et al. 2015). Hence, it is unclear whether preference *per se* or if a combination of preference and target traits differ between sympatric and allopatric populations in both species. Previous research on *Drosophila subquinaria* revealed that sympatric and allopatric populations differ in their cuticular hydrocarbon (CHC) phenotypes and that females from sympatry prefer sympatric CHC phenotypes (Dyer et al. 2014; Rundle and Dyer 2015). However, some literature does suggest that preference might diverge more than signaling traits in regard to reinforcement (Sullivan-Beckers and Cocroft 2010; Debelle et al. 2014; Wheatcroft and Qvarnström 2017). For example, work in birds suggests that reinforcement via discrimination is likely very common and does not require any further diversification of traits (Hudson and Price 2014). This claim is further supported by selection experiments that have successfully altered discrimination windows without noticeably diversifying traits (Kovach 1990). Still, the problem of identifying the relative importance of divergence in preference versus divergence in signaling traits requires direct measurements of these precise traits, and the need to do so is greater when both sexes choose.

Conclusion

In conclusion, we investigated whether patterns of BI in the *Lucania* system matched the predictions of reinforcement when asymmetric postzygotic isolation is present between species. We used a novel experimental design to separately measure mate preference and estimate the contribution to BI for males and females of both species. We found that reinforcement solely due to asymmetric postzygotic isolation does not explain the patterns of BI that we detected. Instead, reinforcement may be acting to increase mate preferences in some groups due to the dramatically reduced fitness of backcrosses or due to the increased costs of hybridization that females incur. Finally, we also highlighted the importance of experimental design when measuring mate preferences and urge future studies to consider the geographic identity of stimulus mates when investigating mate preferences and reinforcement.

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Authors' Contributions

M.E.S.J. and R.C.F. conceptualized the project and reviewed and edited drafts; M.E.S.J. collected and analyzed data and wrote original draft; R.C.F. provided funding.

Conflict of Interest statement

The authors declare no conflicts of interest.

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