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

Michelle E. St. John*, Rebecca C. Fuller
School of Integrative Biology, University of Illinois Urbana-Champaign, Champaign, IL 61820 USA

*Address correspondence to M.E. St. John. E-mail: michelle.e.stjohn@gmail.com

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Abstract

Reinforcement can occur when maladaptive hybridization in sympathy favors the evolution of conspecific preferences and target traits that promote behavioral isolation. In many systems, enhanced behavioral isolation is due to increased female preference for conspecifics. In others, behavioral isolation 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 post-zygotic 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 behavioral isolation (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 sympathy. 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 post-zygotic isolation between species. If true, we predicted that sympatric female *L. goodei* and sympatric male *L. parva* should have lower levels of behavioral isolation compared to their sympatric counterparts, as they produce hybrid offspring with the highest fitness. To address the problem of measuring behavioral isolation when both sexes are choosy, we inferred the contribution to behavioral isolation of each partner using assays where one sex in the mating pair comes from an allopatric population with potentially low preference, while 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 behavioral isolation in sympatry. In the other hybrid cross direction, we found that only female *L. goodei* contribute to behavioral isolation. Sympatric male *L. parva* readily engaged in hybrid spawnings with allopatric *L. goodei* females. These results indicate that both asymmetric post-zygotic isolation and the traditional sex-specific costs to preference likely affect the nature of selection on conspecific preferences and target traits.

**keywords:** 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...
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 sympathy (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 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 behavioral isolation due to male mating preferences (Coyne et al. 1994; Peterson et al. 2005; Servedio 2007; Espino edo 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 behavioral isolation 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; Kvarnemo 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 post-zygotic isolation can also create costs and benefits of choosing and courting that are unique to hybridization between species (Coyne and Orr 2004). Asymmetric post-zygotic isolation is common and occurs when hybrids in one direction (e.g., female A x male B) have lower fitness than hybrids in another direction (e.g., female B x 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 (Pfenning and Simovich 2002; Amtzen et al. 2009) and plants (Coughlan and Willis n.d.; Tiffin et al. 2001; Ramsey et al. 2003). In these scenarios, the expectation is that behavioral isolation 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; Pfenning 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 two 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 sympathy 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 to allopatry for both \textit{L. goodei} and \textit{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 \textit{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 \textit{L. goodei} and female \textit{L. parva} parents, suffer a significant reduction in fitness compared to hybrids formed from female \textit{L. goodei} and male \textit{L. parva} (Fuller 2008). Back-crosses into \textit{L. goodei} also suffer reduced fitness whereas back-crosses into \textit{L. parva} do not. Reinforcement via this process predicts that male \textit{L. goodei} and female \textit{L. parva} should have increased levels of conspecific mate preference compared to female \textit{L. goodei} and male \textit{L. parva} in sympatry. On the other hand, the \textit{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 to their male counterparts. Evidence supporting these predictions is mixed (Berdan and Fuller 2012; Kozak et al. 2015; St. John and Fuller 2018), 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 \textit{Lucania} system (hereafter referred to as behavioral isolation (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 that \textit{L. goodei} females and \textit{L. parva} males from sympatric populations should have lower levels of conspecific preference than \textit{L. parva} females and \textit{L. goodei} males from the same populations. Alternatively, the sex-specific costs of reproduction and hybridization predicts that that conspecific preference may be higher for females of both species in sympatry compared to their male counterparts. We found that male \textit{L. parva} from sympatry contributed less to behavioral isolation 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 \textit{L. goodei}—who we expected to follow the same pattern as male \textit{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 four types of populations for this study: 1) a sympatric population of \textit{L. goodei} from Salt Springs (Marion County, FL; St. John’s river drainage), 2) sympatric populations of \textit{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 \textit{L. goodei} from Blue Springs (Gilchrist County, FL; Santa Fe river drainage), and 4) an allopatric population of \textit{L. parva} from Lake Pontchartrain (St. Tammany County, LA) (For a complete list of sympatric and allopatric populations of \textit{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 authors’ home institution where they resided for the duration of the study period. At the authors’ home institution,
fish were kept in large cattle tanks in an outdoor green house. 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 four 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 one to two 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*. While 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 2018).

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 sympathy 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 sympathy and allopatry (4 mates per individual). Repeated measures of an individual’s mate preference typically provides 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 of 10 blocks of four 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 four 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 five weeks in July and August of 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 one of the four 38-liter tanks in their block. Females remained in their individual tanks for the entirety of the study, while males were moved between female tanks (but stayed in their assigned block). Each individual experienced four no-choice assays, which each lasted for 10 days. Assays began when a male was placed into a female tank along with two top mops (yarn attached to a Styrofoam ball) and two bottom mops (yarn attached to PVC pipe) that served as spawning substrate. The first three 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 seven 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 seventh 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 three and four so that all males were paired for one 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 allopatric *L. parva* from Lake Ponchartrain, 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 two blocks lacked an allopatric *L. parva* female and four 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 behavioral isolation

To make direct comparisons between groups, we used a standardized formula to quantify BI. We used Stalker’s isolation index (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 two 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 sympathy, 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 non-traditional 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 behavioral isolation 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 non-existent conspecific mate preferences (Table 2). However, there is a trend for slightly higher behavioral isolation for crosses between male L. goodei and female L. parva in comparison to crosses between male L. parva and female L. goodei.

Non-traditional crosses show that male L. parva contribute less to behavioral isolation than all other sympatric groups

Non-traditional crosses, involving a combination of animals from sympatric and allopatric populations, allow us to determine which 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 to 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 to 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 vs 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 non-traditional 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 two 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 post-zygotic isolation does not solely explain the pattern of BI in Lucania**

The asymmetric post-zygotic 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 post-zygotic isolation also predicts that female *L. goodei* and male *L. parva* from sympatric populations should have lower levels of behavioral isolation. 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 back-crosses. Fuller (2008) found that F1 hybrids with an *L. goodei* mother exhibited no difference in fitness compared to 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 back-crossed 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 2017). 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 2018). 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 supports both scenarios.

It is also possible that the patterns of behavioral isolation 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 traits (Servedio 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 sympathy has shifted male *L. goodei* target traits, subsequently making them more attractive. However, both traditional and non-traditional 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 has 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 behavioral isolation in sympatry) can be diagnosed by comparing behavioral isolation for sympatric and allopatric animals. However, assessing the relative effect of each sex of each species requires measuring behavioral isolation 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 regards 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 regards to identifying the precise traits that create behavioral isolation. 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 which have successfully altered discrimination windows without noticeably diversifying traits (Kovach 1990). Still, the problem of identifying the relative importance of divergence in preference vs 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 behavioral isolation in the Lucania system matched the predictions of reinforcement when asymmetric post-zygotic 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 that reinforcement solely due to asymmetric post-zygotic 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 back-crosses 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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Author Contributions
M.E.S and R.C.F conceptualized the project, M.E.S collected and analyzed data, M.E.S wrote original draft, M.E.S. and R.C.F reviewed and edited drafts, ad R.C.F provided funding.

Data Accessibility
Data will be uploaded to Dryad upon acceptance.

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

| Response                              | Predictors                        | $\chi^2$ | df | $P$  |
|---------------------------------------|-----------------------------------|----------|----|------|
| 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|
|                                       | ♂ Species: ♂ 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|
Table 2. Estimated levels of behavioral isolation (BI) for traditional and non-traditional crosses for (A) *L. goodei* ♀ x *L. parva* ♂ and (B) *L. parva* ♀ x *L. goodei* ♂. 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. Non-traditional crosses are shown in gray.

|                     | female BI (CL) | male BI (CL) |
|---------------------|----------------|--------------|
| **A) *L. goodei* ♀ x *L. parva* ♂** |                |              |
| sympatric *L. goodei* ♀ x sympatric *L. parva* ♂ | 0.98 (0.93, 1) | 0.97 (0.9, 1) |
| sympatric *L. goodei* ♀ x allopatric *L. parva* ♂ | 0.95 (0.89, 1) | 0.95 (0.82, 1) |
| allopatric *L. goodei* ♀ x sympatric *L. parva* ♂ | 0.41 (-0.4, 0.86) | 0.52 (0.18, 0.89) |
| allopatric *L. goodei* ♀ x allopatric *L. parva* ♂ | 0.33 (-0.24, 1) | 0.54 (-0.12, 1) |
| **B) *L. parva* ♀ x *L. goodei* ♂** |                |              |
| sympatric *L. parva* ♀ x sympatric *L. goodei* ♂ | 0.97 (0.9, 1) | 0.98 (0.93, 1) |
| sympatric *L. parva* ♀ x allopatric *L. goodei* ♂ | 0.95 (0.88, 1) | 0.94 (0.83, 1) |
| allopatric *L. parva* ♀ x sympatric *L. goodei* ♂ | 0.97 (0.88, 1) | 0.97 (0.92, 1) |
| allopatric *L. parva* ♀ x allopatric *L. goodei* ♂ | 0.68 (0.15, 0.99) | 0.52 (-0.00063, 0.97) |
### A) Female Behavioral Isolation

![Figure A](image)

### B) Female Behavioral Isolation

| Pair Type       | Female Geography | Female Species | Male Geography  | Male Species | Male Species Pair Type |
|-----------------|------------------|----------------|----------------|--------------|------------------------|
| Sympatric       | ♀ A x sympatric B| A Sympatric    | A Sympatric    | A            | Conspecific            |
| Sympatric       | ♀ A x Allopatric B| A Sympatric    | A Allopatric   | B            | Heterospecific         |
| Allopatric      | ♀ A x Sympatric A| A Allopatric   | A Sympatric    | A            | Conspecific            |
| Allopatric      | ♀ A x Allopatric A| A Allopatric   | A Allopatric   | A            | Heterospecific         |

### C) Male Behavioral Isolation

| Pair Type       | Female Geography | Female Species | Male Geography  | Male Species | Male Species Pair Type |
|-----------------|------------------|----------------|----------------|--------------|------------------------|
| Sympatric       | ♀ B x sympatric A| B Sympatric    | A Sympatric    | A            | Heterospecific         |
| Sympatric       | ♀ B x Allopatric A| B Sympatric    | A Sympatric    | B            | Conspecific            |
| Allopatric      | ♀ B x Sympatric A| B Allopatric   | A Sympatric    | A            | Heterospecific         |
| Allopatric      | ♀ B x Allopatric A| B Allopatric   | A Allopatric   | B            | Conspecific            |
| Allopatric      | ♀ B x Sympatric A| B Allopatric   | B Allopatric   | A            | Heterospecific         |
| Allopatric      | ♀ B x Allopatric A| B Allopatric   | B Allopatric   | B            | Conspecific            |
| BI: Allopatric ♂ A x | Allopatric | A | Allopatric | A | Conspecific |
|----------------------|------------|---|------------|---|-------------|
| Sympatric ♀ B        | **Sympatric** | B | Allopatric | A | Heterospecific |
| BI: Allopatric ♂ A x | Allopatric | A | Allopatric | A | Conspecific |
| Allopatric ♀ B       | **Allopatric** | B | Allopatric | A | Heterospecific |
| BI: Sympatric ♂ B x  | **Sympatric** | A | **Sympatric** | B | Heterospecific |
| Sympatric ♀ A        | **Sympatric** | B | **Sympatric** | B | Conspecific |
| BI: Sympatric ♂ B x  | **Allopatric** | A | **Sympatric** | B | Heterospecific |
| Allopatric ♀ A       | **Sympatric** | B | **Sympatric** | B | Conspecific |
| BI: Allopatric ♂ B x  | **Allopatric** | A | **Allopatric** | B | Heterospecific |
| Allopatric ♀ A       | **Allopatric** | B | **Allopatric** | B | Conspecific |

**Figure 1.** (A) Species A and B and their geographic relationships. (B) Crosses used to measure behavioral isolation for females as a function of sympatry and allopatry. (C) Crosses used to measure behavioral isolation for males as a function of sympatry and allopatry. Traditional crosses used to diagnose RCD in white. Non-traditional 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) represents the 16 pairs from the female perspective. B) represents the 16 pairs from the male perspective. The x-axis shows species and geographic designations, while boxplot colors indicate species and geographic designations of the mate.