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GENETIC INCOMPATIBILITIES IN KILLIFISH AND THE ROLE OF ENVIRONMENT

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Measuring reproductive isolation across multiple generations and environments is a key endeavor in speciation research because it indicates which isolating barriers currently prevent introgression and the extent to which they are intrinsic versus environmentally dependent. Here, I present data from several crosses (parental crosses, F1s, F2s, back-crosses) between two species of killifish (*Lucania goodei* and *L. parva*) that have diverged along a salinity gradient (*L. goodei*—freshwater, *L. parva*—euryhaline). Offspring were raised under high and low salinity to test for (1) extrinsic isolation, (2) intrinsic isolation manifested through genetic incompatibilities, and (3) environmentally dependent genetic incompatibilities. I found evidence for both intrinsic and extrinsic isolation, but no evidence for environmentally dependent genetic incompatibilities. The presence of extrinsic and intrinsic isolation varied among fitness measures, and all forms of reproductive isolation were asymmetric. Early egg survival was independent of salinity, but demonstrated pronounced intrinsic isolation. Both extrinsic and intrinsic isolation existed for egg hatching and survival of fry to the eating stage. Unfortunately, the order in which extrinsic and intrinsic isolation arose is unresolved. Understanding the extent to which adaptation to salinity creates multiple forms of reproductive isolation is critical for understanding diversification in many fish taxa.

KEY WORDS: **Epistasis, F2 breakdown, Fundulidae, genetic incompatibility, joint scaling analysis, line cross analysis, osmoregulation, reproductive isolation.**

The common observation that closely related species live in different habitat types and possess apparent adaptations to those habitats has led to the supposition that differential natural selection between populations is important in speciation (Dobzhansky 1951; reviewed in Schluter 2000, 2001; Coyne and Orr 2004; Rundle and Nosil 2005; Funk et al. 2006). Ecological speciation relies on divergence among populations as a result of adaptation to different environments which results in reproductive isolation. The classic evidence for ecological speciation is the finding that hybrids and back-crosses have reduced fitness relative to parentals in the parents' native habitats. For a given parental habitat, there should be a positive relationship between fitness and the proportion of parental alleles from the native parent. This is also known as ecologically dependent postzygotic isolation (hereafter referred to as extrinsic isolation for brevity) (Rundle and Whitlock 2001). Extrinsic isolation provides unambiguous support for ecological selection causing reproductive isolation (Hatfield and Schluter 1999; Via et al. 2000; Rundle 2002; Dettman et al. 2007).

Intrinsic isolation occurs when hybrid offspring are less fit than the parental species in all environments (review in Burke and Arnold 2001) and can involve reduced fitness in the F1 and/or F2 generations as well as reduced fitness in back-crosses. Unlike extrinsic isolation (which provides unambiguous support for ecological selection), all forms of speciation can result in intrinsic isolation—including both ecological and nonecological speciation (Dobzhansky 1951; Orr 1995; Schluter 2000; Gavrilets 2003; Dettman et al. 2007, 2008). Nonecological speciation includes forms of selection unrelated to divergent parental environments as well as speciation by polyploidization, hybridization, and genetic drift (Rundle and Nosil 2005). Rundle and Nosil (2005) argue that intrinsic isolation arises more quickly under divergent selection—which includes ecological selection (see Bordenstein

and Drapeau 2001; Coyne and Orr 2004 for discussions of the role of selection on intrinsic isolation). Studies in which the alleles responsible for postzygotic isolation have been identified have also documented positive selection on those alleles (Ting et al. 1998; Presgraves et al. 2003; Barbash et al. 2004; Brideau et al. 2006), but have generally been unable to identify the exact nature of selection.

Historically, studies of intrinsic and extrinsic isolation have differed in their experimental approaches. Studies of intrinsic isolation have focused on determining whether particular types of genes and/or particular types of genetic architectures cause genetic incompatibilities (Rieseberg et al. 1995, 1999; Fishman et al. 2001; Hawthorne and Via 2001; Sweigart et al. 2006; Ellison and Burton 2008; see Coyne and Orr 2004 for a review). Until recently, fewer studies focused on determining the genetic architecture underlying extrinsic isolation (but see Bradshaw et al. 1995; Filchak et al. 2000; Gross et al. 2004). This may have stemmed in part from the fact that, theoretically, extrinsic isolation need not involve epistasis (Coyne and Orr 2004, p. 255). A purely additive model of gene action can result in extrinsic isolation provided that there are no intermediate environments in which hybrids have higher fitness than the two parental species (Rundle and Whitlock 2001; Rundle 2002; Coyne and Orr 2004). However, adaptation in different environments can also lead to the fixation of alleles that are incompatible with one another in either F1 or F2 hybrids (Dobzhansky 1951; Schluter 2000; Bordenstein and Drapeau 2001; Gavrilets 2003; Dettman et al. 2007, 2008).

An increasing number of studies have indicated that genetic incompatibilities (i.e., negative epistatic interactions) also play a role in extrinsic isolation (Bordenstein and Drapeau 2001; Campbell and Waser 2001, 2007; Rawson and Burton 2002; Willett and Burton 2003; Demuth and Wade 2007a,b; Rogers and Bernatchez 2006). Environmentally dependent genetic incompatibilities may be underappreciated in the scientific literature because many genetic studies (particularly those on model organisms) only measure traits and fitness in a single environment. Like intrinsic isolation, environmentally dependent genetic incompatibilities can result from either ecological or nonecological speciation.

Delineating between extrinsic and intrinsic isolation is challenging. Ideally, one would unambiguously identify the alleles contributing to reproductive isolation and would determine whether these vary with environmental conditions (Bordenstein and Drapeau 2001; Willett and Burton 2003). In lieu of this, one can use the methodology of Rundle and Whitlock (2001, described below) to test for the presence of an interaction between environment and the proportion of alleles derived from the native parent (additive genetic effect $\times$ environment) and whether each species has higher fitness than the other in its native habitat with hybrids and back-crosses displaying intermediate fitness as a function of the proportion of genes inherited from the native species (Rundle 2002). In this study, intrinsic isolation is inferred from negative epistatic interactions that reduce fitness (e.g., F2 breakdown) and are independent of environmental conditions. Interpreting environmentally dependent genetic incompatibilities is particularly problematic because they can arise from two different mechanisms. First, different sets of alleles may be required for different physiological functions in different environments and, as a result, genetic incompatibilities are environment specific. Second, the same sets of alleles may contribute to genetic incompatibilities in different environments, but their magnitude may differ with higher levels of reproductive isolation occurring under more stressful habitats. Despite these issues, estimating the genetic architecture underlying reproductive isolation is a critical first step for understanding the evolution of genetic incompatibilities (Fitzpatrick 2008a), the extent to which they vary with environment (Demuth and Wade 2007a), and the extent to which reproductive isolation may be a function of environmental conditions (Rundle and Whitlock 2001).

This study has three specific goals. The first goal is to test for extrinsic isolation as a function of environment (additive genetic $\times$ environment effect). The second goal is to test for genetic incompatibilities (overall epistatic effects). The third goal is to test whether the expression of genetic incompatibilities is dependent on environmental conditions by comparing early life-history stages among crosses under different salinity environments (epistatic effects that vary with environmental conditions). To do this, I apply line cross analysis to a series of crosses between two killifish species that have been raised under different salinity treatments. The goal of line cross analysis is to determine the (1) additive genetic effects, (2) dominance effects, (3) epistatic effects, (4) environmental effects, and (5) the extent to which the genetic effects vary with environment (Rundle and Whitlock 2001). This is done by regressing trait values on coefficients of determination that describe the expected contribution from each genetic effect. This method has frequently been used to describe genetic effects among populations within species (Armbruster et al. 1997; Edmands and Burton 1999; Galloway and Fenster 2001; Miller et al. 2003; Fox et al. 2004a,b; Demuth and Wade 2007a,b), and has increasingly been used to describe genetic effects among species (Fritz et al. 2006; Rego et al. 2007; see Fitzpatrick 2008b for a similar approach fitting genetic effects to the Turelli and Orr 2000 model), but has been used less frequently to discern the relative contributions of extrinsic and intrinsic isolation (Czesak et al. 2004).

In this study, I test for extrinsic isolation, genetic incompatibilities, and whether the expression of genetic incompatibilities is dependent upon environmental conditions in hybrids and back-crosses between two closely related killifish, *Lucania goodei* and *L. parva*, which have diverged across a salinity gradient. Salinity

is a very important variable for nearly all aquatic organisms. The ecological transition from freshwater to seawater that occurs along sea coasts poses a variety of challenges to organisms and is marked by rapid shifts in aquatic communities (Gunter 1945, 1950a,b; Davis 1955; Godfrey and Wooten 1979, 1981). This shift in abundance reflects the differing abilities of species to deal with the osmotic demands posed by fresh versus marine water. Freshwater teleosts need to keep excess water out of their bodies while retaining salts whereas marine forms need to extricate salt, but retain water (for a review see Evans et al. 2005). Within the Actinopterygii, most fish families (70%) are found exclusively in either marine or freshwater (Nelson 2006) suggesting that the evolutionary transition between fresh and salt water is difficult in some groups (Lee and Bell 1999). Yet other groups contain freshwater, brackish, and marine species (e.g., Gasterosteiformes, Fundulidae, Atherinopsidae) with closely related species differing primarily in habitat salinity. These patterns indicate an important role of salinity in reproductive isolation and diversification of teleost fish (Mank and Avise 2006).

Lucania goodei and *L. parva* are an ideal system for studying the role of salinity on reproductive isolation. Although they differ slightly in morphology and coloration (Page and Burr 1991; Fuller 2002; Fuller and Travis 2004), the most striking phenotypic differences involve salinity tolerance. *Lucania goodei* is found primarily in freshwater sites in Florida. *Lucania parva* is euryhaline and can be found in fresh, brackish, and marine habitats ranging from Cape Cod around the Florida peninsula and through the Gulf of Mexico coast (Lee et al. 1980). In a review of over 1400 museum records from the University of Florida, Fuller and Noa (2008) found that 93% of *L. goodei* populations were classified as freshwater with the remaining classified as brackish. *Lucania parva* was found readily in all three habitat types with 23% of populations in freshwater, 46% in brackish water, and 31% in marine water. These differences in habitat have led to differential adaptation to salinity. *Lucania goodei* eggs have low hatching success in high salinity (Fuller 2008), and *L. goodei* overwinter adult survival decreases as salinity increases (Fuller et al. 2007). The opposite pattern is seen in *L. parva* where overwinter, adult survival increases as salinity increases (Fuller et al. 2007). In addition, salinity has been shown to affect the outcome of competition between these two species (Dunson and Travis 1991).

Lucania goodei and *L. parva* are closely related. Both allozyme and molecular data suggest that *L. goodei* and *L. parva* are sister species (Duggins et al. 1983 ; T. Hrbek, pers. comm.). *Lucania goodei* and *L. parva* are sympatric over a sizable portion of their range in Florida. In Florida, approximately 15% of *L. goodei* populations are sympatric with *L. parva*, and 17% of *L. parva* populations are sympatric with *L. goodei* (Fuller and Noa, 2008). Of the sympatric sites, two-thirds are found in freshwater and one-third is found in brackish water. Furthermore, there is evidence that hybrids do occur at low frequencies. Hubbs et al. (1943) report hybrid fish that were intermediate in meristic characters between *L. goodei* and *L. parva*. Hybrids were detected in four populations that occurred in the St. Mark's National Wildlife Refuge, Wakulla, Co., Florida. Hybrids were rare and were less than 1% of the combined *Lucania* population. In our laboratory, we are currently using molecular markers to further measure the degree of hybridization in natural populations.

Methods

The goals of this experiment were to test for (1) extrinsic isolation, (2) intrinsic isolation manifested by genetic incompatibilities, and (3) whether the expression of genetic incompatibilities varied with salinity. To do this, I compared survival across three early life-history stages among F1 crosses, F2 crosses, back-crosses, and conspecific parental crosses of *L. goodei* and *L. parva* that had been raised under high and low salinity. This experiment involved using animals from three separate breeding studies. Below, I describe each of the breeding studies and the manner in which the animals were used.

2005 BREEDING STUDY—CREATION OF PARENTS

I used *L. goodei, L. parva,* and both types of F1 hybrids as parents to create F2 hybrids, back-crosses, and conspecific crosses that served as controls in the "2006 Breeding Study 1" (see below). Here, I briefly describe the 2005 breeding study in which I generated these parents. In May–July 2005, Fuller and colleagues performed four types of crosses (Fuller et al. 2007). We performed conspecific *L. goodei*, conspecific *L. parva*, and both types of F1 crosses ($F1_P$: *L. parva* ♂ × *L. goodei* ♀; $F1_G$: *L. goodei* ♂ × *L. parva* ♀ where P and G refer to sire identity) using wild caught animals. *Lucania parva* were collected from Lighthouse Pond on the St. Mark's National Wildlife Refuge and from the Three-Finger's Site on the lower Wakulla River (Wakulla Co., Florida). *Lucania goodei* were collected from the Upper Bridge Site and from the Three-Fingers Site both of which are on the Wakulla River (Wakulla Co., Florida, see Fuller et al. 2007 for further details on these populations). Upper Bridge and Three-Fingers are freshwater sites. Lighthouse Pond is brackish. *Lucania goodei* and *L. parva* are sympatric at Three-Fingers.

All male/female pairs were maintained in 19-l aquaria with water at 2 ppt. The resulting eggs were collected and raised in the laboratory in 0, 2, 4, or 8 ppt salinity until the larval stage. In August 2005, these animals were transferred to outdoor cattle tanks behind the Mission Road Greenhouse at Florida State University where they were raised to adulthood. *Lucania goodei* and *L. parva* were raised in the same salinities (0, 2, 4, or 8 ppt) at which they had been kept during larval development. *Lucania*

goodei were pooled between the Upper Bridge and Three-Fingers populations. *Lucania parva* were pooled between the Lighthouse Pond and Three-Fingers populations. Due to a shortage of cattle tanks, all F1 hybrids were raised at 2 ppt. In March 2006, the cattle tanks were censused, and the animals were transported back to the University of Illinois. Throughout this article, I use $F1_P$ and $F1_G$ to refer to data from the original F1 crosses from Fuller et al. (2007). P and G refer to the identity of the father in the original F1 cross (P—*L. parva*, G—*L. goodei*). For sires and dams used in F2 crosses and back-crosses, I refer to Hybrid P and Hybrid G that are the adult offspring from $F1_P$ and $F1_G$.

2006 BREEDING STUDY 1—F2, BACK-CROSSES, AND CONSPECIFIC CROSSES

For these crosses, I used the animals generated from the 2005 breeding study as parents. I performed 12 types of crosses: two parental conspecific crosses (*L. goodei* ♀ × *L. goodei* ♂ $N = 4$, *L. parva* ♀ × *L. parva* ♂ $N = 4$), two F2 crosses (Hybrid P ♀ × Hybrid G ♂ $N = 4$, Hybrid G ♀ × Hybrid P ♂ $N = 4$), and eight back-crosses (*L. goodei* ♀ × Hybrid P ♂ $N = 2$, *L. goodei* ♀ × Hybrid G ♂ $N = 2$, *L. parva* ♀ × Hybrid P ♂ $N = 2$, *L. parva* ♀ × Hybrid G ♂ $N = 2$, Hybrid P ♀ × *L. goodei* ♂ $N = 2$, Hybrid P ♀ × *L. parva* ♂ $N = 2$, Hybrid G ♀ × *L. goodei* ♂ $N = 2$, Hybrid G ♀ × *L. parva* ♂ $N = 2$). For each cross, I placed one male and one female in a 38 L aquarium containing conditioned city water at 1.5–2 ppt salinity. City water was treated with Start Rite (Jungle Laboratories Corporation, Cibolo TX) water conditioner that removes all chlorine. The water was then filtered to remove ammonia (resulting from chloramine). This resulted in hard, freshwater that is suitable for *Lucania*. Animals were fed frozen adult brine shrimp. Lights were maintained on a 14L:10D light ratio.

2006 BREEDING STUDY 2—F1 AND CONSPECIFIC CROSSES

To more fully elucidate the genetic and environmental basis of hybrid incompatibilities, I pooled the above crosses with a series of F1 and parental crosses generated by Fuller et al. (2007). In April–June 2006, Fuller et al. (2007) conducted four types of crosses: two parental crosses and two F1 crosses ($F1_P$: *L. parva* ♂ × *L. goodei* ♀; $F1_G$: *L. goodei* ♂ × *L. parva* ♀). These crosses duplicated those conducted in 2005 (see above), but were conducted at the University of Illinois. Both 2006 breeding studies were conducted at the exactly same time and used identical methods with two exceptions. First, "2006 Breeding Study 1" crosses were performed in 38 L aquaria whereas the "2006 Breeding Study 2" crosses were performed in 19 L aquaria. Second, the "2006 Breeding Study 1" used animals from the "2005 Breeding Study" as parents whereas the "2006 Breeding study 2" used wild-caught animals. *Lucania parva* were collected at Lighthouse Pond and at the Lower Bridge site on the Wakulla River (Wakulla Co., Florida). *Lucania goodei* were collected at the Upper Bridge and Lower Bridge sites on the Wakulla River (Wakulla Co., Florida, see Fuller et al. 2007 for further details on these populations). Lower Bridge is a freshwater site and is sympatric for the two species. All other methods were identical.

PHENOTYPIC ASSAYS

To encourage spawning, each aquarium was stocked with four yarn mops (two with floats and two with sinks) that provided spawning substrates at various depths. Spawning substrates were checked for eggs once every two to three days. Eggs were removed and placed in tubs with water set at 0, 2, 4, 8, 15, or 20 ppt salinity. Eggs were treated with a dilute amount of methylene blue to prevent fungus infections. All aquaria were checked for eggs from April 24, 2006 until June 2, 2006. After June 2, I continued to check for eggs for pairs of fish that had not produced a minimum of 10 eggs for each salinity treatment (60 eggs total). During the experiment, five adult animals died (two *L. parva* ♀, one *L. goodei* ♂, one Hybrid G ♀, one Hybrid G ♂). These animals were immediately replaced and crosses continued. One cross between an *L. parva* ♀ and a Hybrid G ♂ produced eggs for the 0, 2, 4, and 8 ppt salinity treatments, but no eggs for the 15 and 20 ppt treatments. One conspecific *L. goodei* cross produced no eggs.

Egg status was checked once every two to three days. Dead eggs are readily detectable because they deteriorate and absorb the methylene blue dye. I also recorded the number of eggs that hatched and the number of fry that survived to the eating stage. I diagnosed when fry had consumed *Artemia* by the presence of a visible, pink bolus of food in the gut. From these data, I calculated survival over three stages. First, I calculated early egg survival as the survival of eggs from day 0 to day 5 postfertilization. Early egg survival encompasses both fertilization success and the early stages of development. Second, I measured hatching success as the number of eggs that lived from day 5 until hatching. Third, I measured the proportion of fry that survived until they were capable of independent foraging. Raw data for early egg survival and hatching success are presented in Supporting Table S1.

STATISTICAL ANALYSES

I first analyzed the effects of cross, salinity, and the interaction between cross and salinity on the three early life-history variables (early egg survival, hatching success, survival to the eating stage). I used a logit analysis in SAS Proc Genmod (SAS, Cary, NC) assuming a binomial distribution. For all analyses, I used the dscale option in Proc Genmod that corrects for overdispersion (i.e., deviance/df >1) and produces a more conservative model. The log likelihood and chi-square statistics were scaled by the deviance/df. Standard errors of estimates were scaled by

(deviance/df)$^{1/2}$. To examine the overall effects of cross, salinity, and the interaction between cross and salinity, I performed a type 3 analysis that examines the effects of the model with and without each term. For early egg survival, I considered 14 different types of crosses—two parental crosses, two F1 crosses, two F2 crosses, and eight types of back-cross. For hatching success and survival to the eating stage, my sample size was smaller, and I was unable to consider the direction of each type of cross. I therefore collapsed the cross types into the following six categories: *L. goodei* conspecific, back-cross to *L. goodei*, F1, F2, back-cross to *L. parva*, *L. parva* conspecific.

I performed subsequent post hoc tests to examine differences among treatments using least-squares means of logit values. For the analysis of early egg survival, I only considered clutches that initially contained at least six eggs. For the analysis of the proportion of eggs that hatched, I only considered clutches that had a minimum of six eggs that survived the early stage. For the analysis of survival to the eating stage, I only considered clutches that had a minimum of six eggs that hatched.

An initial analysis indicated that there were no statistically significant differences between 0, 2, 4, or 8 ppt nor were there differences between 15 and 20 ppt, but there were differences between eggs raised at low salinities (0, 2, 4, or 8 ppt) and those raised at high salinities (15 or 20 ppt). I therefore pooled the salinity treatments into low salinity (i.e., 0, 2, 4, or 8 ppt) and high salinity (i.e., 15 or 20 ppt) treatments. This greatly increased the power of the analysis and allowed for more precise estimates of means and standard errors for each treatment combination. Results from the analysis were robust and produced similar results to a general linear model on arcsine square-root transformed proportions. Raw means and standard errors are shown in all figures. *P* values for all post-hoc tests are two-tailed.

LINE CROSS ANALYSIS

I performed line cross analysis to estimate the additive and dominance effects, three types of epistatic effects, the effects of the environment, and the extent to which the genetic effects varied with environment (Lynch 1991; Rundle and Whitlock 2001; Demuth and Wade 2005, 2007a,b; Fitzpatrick 2008a). The analysis involved assigning coefficients of determination to each cross that describe the expected contribution from each genetic effect. For example, for the additive effect, one parental species (*L. goodei*) is assigned a coefficient of 1; the other (*L. parva*) was assigned −1; F1s and F2s were expected to be completely intermediate under an additive model and were assigned 0; back-crosses to *L. goodei* were 0.5; back-crosses to *L. parva* are −0.5. The effect of dominance describes dominant effects between loci derived from the two parental species. The effect is greatest in the F1s where animals are heterozygous at all loci, absent in the parental crosses, and intermediate in the F2s and back-crosses where half of the loci are expected to be heterozygous. I also estimated the effects of three types of epistasis—additive × additive epistasis ($I_{\alpha\alpha}$), additive × dominance epistasis ($K_{\alpha\delta}$), dominance × dominance epistasis ($J_{\delta\delta}$). These effects have been thoroughly described elsewhere (Lynch 1991; Demuth and Wade 2005). It is worth noting that there are slight differences between the Demuth and Wade model (2005), and the Lynch model (1991), which have been thoroughly delineated by Fitzpatrick (2008a). Here, I follow the method described by Rundle and Whitlock (2001), which is an expansion of the Lynch (1991) model. The coefficients of determination are listed in Supporting Table S2 (see Lynch 1991; Lynch and Walsh 1998; Rundle and Whitlock 2001 for the derivation of coefficients). The 12 coefficients represent the mean and the effect of environment (E), the genetic effects of additivity, dominance, $I_{\alpha\alpha}$, $K_{\alpha\delta}$, $J_{\delta\delta}$, and the genetic interactions with environment—additive × E, dominance × E, $I_{\alpha\alpha}$ × E, $K_{\alpha\delta}$ × E, and $J_{\delta\delta}$ × E.

I used logistic regression to (1) determine which effects should be included in the best-fit model and to (2) estimate the magnitude and standard errors for each of the effects. I chose to use a logistic regression because the data are binomial and because a preliminary analysis using general linear models occasionally resulted in predicted means that were nonsensical (i.e., probability of surviving greater than 1 or less than 0). All models included an intercept (i.e., a nonzero population mean).

I first considered a model that was consistent with extrinsic isolation and included the additive genetic, environmental, and additive genetic × environmental effects. I then checked whether each of the three effects deserved to remain in the model by comparing models with and without each term. Provided that the term improved the fit of the model, I determined whether the improved fit was statistically significant by taking the difference of two times the log likelihood between the two models and determining the significance using the chi-square probability and assuming a single degree of freedom (Lynch and Walsh 1998). I then proceeded to test the effects of dominance and each type of epistasis as well as their interactions with the environmental treatments using the procedure described above. I performed all analyses in SAS using Proc Genmod using the dscale option.

For best-fit models that were consistent with both extrinsic isolation (i.e., a significant additive × environment effect) and intrinsic isolation (i.e., a significant effect of epistasis), I examined the contribution of each effect on the distribution of the data by comparing the predicted means from a model with only extrinsic isolation with a model that also included epistatic effects. The predicted means were determined by calculating the anti-logit of X_{beta}. X_{beta} is the sum of the products of the estimate for each effect times the corresponding coefficient of determination for each cross type.

Results

EARLY EGG SURVIVAL

All three measures of offspring fitness varied among cross types but differed with respect to the effects of environment and the interaction between cross and environment (Table 1 A–C). Early egg survival was overwhelmingly affected by cross, but showed little effect of environment or cross by environment (Table 1A). Early egg survival encompasses both fertilization and egg survival through day 5 postfertilization. Figure 1 shows early egg survival as a function of cross. Genetic incompatibilities were manifested in the low survival of all crosses involving hybrid G males (recall that hybrid G = *L. goodei* ♂ × *L. parva* ♀ and hybrid P = *L. parva* ♂ × *L. goodei* ♀). There are five critical comparisons indicating that crosses involving hybrid G males have low early egg survival. First, in crosses involving hybrid P females, early egg survival with hybrid G males was half that with either *L. goodei* or *L. parva* males (Fig. 1, $P < 0.0005$ in all comparisons). This reduction in fitness could in theory be attributable to simple F2 breakdown. However, F2 crosses involving hybrid G males had lower survival than those involving hybrid P males ($\chi^2_1 = 9.89$, $P = 0.0017$). Also, early egg survival did not differ between the three cross types involving hybrid G males ($P > 0.44$ in all comparisons). Most importantly, back-crosses involving hybrid G males had reduced hatching success. In crosses with *L. goodei* females, early egg survival with hybrid G males was approximately half that with *L. goodei* males, hybrid P males, or *L. parva* males ($P < 0.02$ in all comparisons). Similarly, in crosses with *L. parva* females, early egg survival with hybrid G males was half that with either *L. parva*, hybrid P, or *L. goodei* males ($P < 0.02$ in all comparisons). The two types of F1 males should be genetically identical with exception to their sex chromosomes and mitochondria. The fact that hybrid G males have reduced early egg survival regardless of the identity of the parental female suggests that genetic incompatibilities are a property of hybrid G males themselves and not an interaction between hybrid G males and the genetic properties of the female.

Hybrid P males did not show the same pattern. Hybrid P males had high early egg survival when back-crossed to either *L. goodei* or *L. parva* females. In F2 crosses with hybrid G females, hybrid P males had slightly reduced hatching success (~75%) in comparison to back-crosses with either *L. goodei* or *L. parva* males (Fig. 1, $\chi^2_1 = 6.15$, $P = 0.0131$, $\chi^2_1 = 2.56$, $P = 0.0313$). F2 crosses involving hybrid P males had early egg survival approximately 88% that of $F1_P$ and $F1_G$ crosses, but these differences were not statistically significant ($\chi^2_1 = 0.38$, $P = 0.5368$; $\chi^2_1 = 1.86$, $P = 0.1725$). There was no evidence for genetic

Table 1. Logit analyses for the effects of cross, salinity, and their interaction on three early life-history measures. Because of overdispersion, log-likelihood and χ^2 statistics were scaled by the deviance/df resulting in a more conservative model. The number of cross types for (A) probability of early egg survival is higher because this analysis considered the direction of the crosses for F1, F2, and back-crosses. The analyses for (B) probability of hatching and (C) probability of surviving to the eating stage have fewer cross types because the direction of the cross is ignored for F1, F2, and back-crosses.

Source	df	χ^2	P
(A) Probability of early egg survival			
Cross	13	133.4	<0.0001
Salinity	1	0.1	0.7834
Cross×salinity	13	10.0	0.6919
log likelihood=−572.8, deviance/df=4.0			
(B) Probability of hatching			
Cross	5	142.4	<0.0001
Salinity	1	16.6	<0.0001
Cross×salinity	5	76.6	<0.0001
log likelihood=−596.2, deviance/df=3.3			
(C) Probability of surviving to the eating stage			
Cross	5	17.5	0.0036
Salinity	1	2.6	0.1099
Cross×salinity	5	24.8	<0.0001
log likelihood=−234.7, deviance/df=4.9			

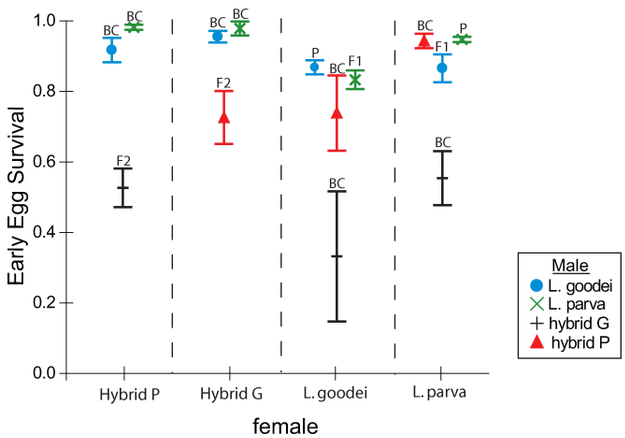

Figure 1. Early egg survival as a function of dam and sire identity. Raw means and standard errors are shown. Early egg survival is the survival from day 0 postspawning to day 5. Parental crosses (P), back-crosses (BC), F1 crosses (F1), and F2 crosses (F2) are indicated on the figure. Sample sizes as follows: Hybrid P ♀ × Hybrid G ♂ *N* = 7, Hybrid P ♀ × *L. goodei* ♂ *N* = 4, Hybrid P ♀ × *L. parva* ♂ *N* = 4, Hybrid G ♀ × Hybrid P ♂ *N* = 8, Hybrid G ♀ × *L. goodei* ♂ *N* = 4, Hybrid G ♀ × *L. parva* ♂ *N* = 4, *L. goodei* ♀ × *L. goodei* ♂ *N* = 29, *L. goodei* ♀ × Hybrid P ♂ *N* = 4, *L. goodei* ♀ × Hybrid G ♂ *N* = 4, *L. goodei* ♀ × *L. parva* ♂ *N* = 13, *L. parva* ♀ × *L. parva* ♂ *N* = 34, *L. parva* ♀ × Hybrid P ♂ *N* = 4, *L. parva* ♀ × Hybrid G ♂ *N* = 3, *L. parva* ♀ × *L. goodei* ♂ *N* = 6.

incompatibilities in early egg survival involving F1 females back-crossed to either parental species ($P > 0.07$ in all comparisons).

The reduction in the fitness of F2s and of some types of back-crosses was attributable to intrinsic isolation and the combined effects of epistasis and dominance. Epistasis and dominance accounted for large effects on the distribution of the data. An examination of the predicted means for a model without dominance and a model without epistasis indicated that reductions in fitness in the F2 stage were attributable to the effects of dominance and epistasis (data not shown). Table 2A shows the estimates for the best-fit model for the probability of early egg survival. The combined effects of digenic epistasis accounted for 69% of the predicted effects. The best-fit model also included the effect of $J_{\delta\delta}$ × environment, although this effect was not different from zero. Unfortunately, the analysis for early egg survival could not account for the effects of the direction of the cross and therefore could not account for the reduced fitness of crosses involving hybrid G males because the coefficients of determination did not differ as a function of cross direction (Supporting Table S2).

Table 2. Logistic regression of early life-history measures on coefficients of determination. Because of overdispersion, the log-likelihood and chi-square statistics were scaled by the deviance/df, and the standard error (SE) of the estimates (β) were scaled by (deviance/df)$^{1/2}$ resulting in more conservative results. *P* is the probability that the estimates (β) are significantly different from zero. Following Demuth and Wade (2007a), the proportional effects represent the proportion of the total effects that are due to each specific type of effect (i.e., absolute value of additive effect/sum of absolute values for all genetic effects).

Source	β	SE	*P*	Proportional effects
(A) Probability of early egg survival				
Intercept	0.75	0.19	<0.0001	
Additive	−1.01	0.42	0.0151	0.09
Dominance	2.52	0.59	<0.0001	0.22
$I_{\alpha\alpha}$	5.56	1.13	<0.0001	0.49
$K_{\alpha\delta}$	−1.42	0.51	0.0052	0.12
$J_{\delta\delta}$	−0.61	0.44	0.1689	0.05
$J_{\delta\delta}$×environment	−0.3	0.2	0.1383	0.03
log likelihood=−376.6, deviance/df=6.7				
(B) Probability of hatching				
Intercept	1.23	0.12	<0.0001	
Additive	−1.23	0.14	<0.0001	0.37
Environment (E)	0.51	0.10	<0.0001	0.14
Additive×E	1.01	0.14	<0.0001	0.29
$J_{\delta\delta}$	0.71	0.16	<0.0001	0.20
log likelihood=−543.3, deviance/df=3.7				
(C) Probability of surviving to the eating stage.				
Intercept	2.05	0.21	<0.0001	
Additive	−0.98	0.27	0.0004	0.33
Environment (E)	0.34	0.17	0.0484	0.11
Additive×E	1.11	0.27	<0.0001	0.37
$J_{\delta\delta}$	0.54	0.27	0.0424	0.18
log likelihood=−235.6, deviance/df=5.0				

HATCHING SUCCESS

Hatching success was affected by cross, salinity, and the interaction between cross and salinity (Table 1B). Figure 2 shows hatching success for all cross types in both low and high salinity treatments. The largest differences among cross types occurred in the high salinity treatment. All crosses involving *L. parva* (i.e., conspecific *L. parva* crosses, back-crosses to *L. parva*, and F1 crosses) had high hatching success in the high salinity treatment. In contrast, the hatching success of *L. goodei* conspecific crosses was 25% that of *L. parva* conspecific crosses in the high salinity treatment. Hatching success of F1s was 15% lower than *L. parva* ($\chi^2_1 = 3.05$, $P = 0.0809$) but was 3.3 times greater than *L. goodei*

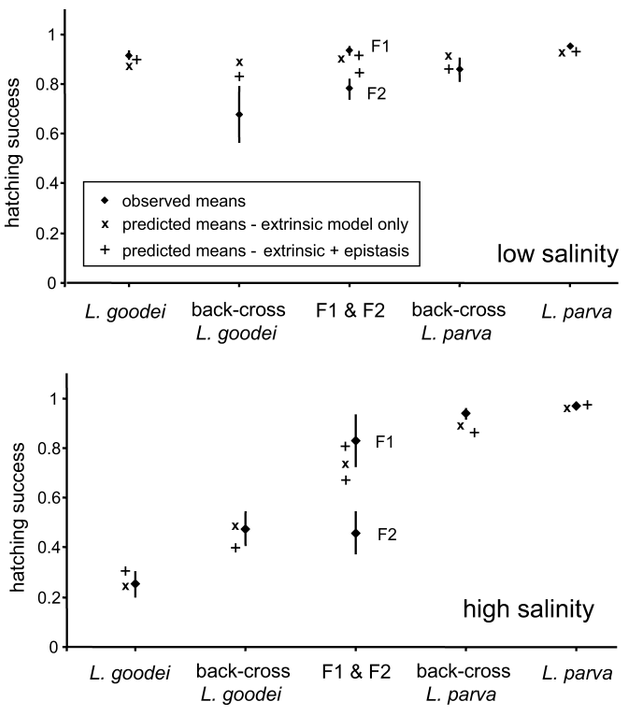

Figure 2. Hatching success in low and high salinity environments as a function of cross. Means and standard errors are shown. Sample sizes are as follows: low salinity—*L. goodei* *N* = 17, back-cross to *L. goodei* *N* = 8, F1 *N* = 13, F2 *N* = 8, back-cross to *L. parva* *N* = 8, *L. parva* *N* = 18; high salinity—*L. goodei* *N* = 12, back-cross to *L. goodei* *N* = 7, F1 *N* = 6, F2 *N* = 7, back-cross to *L. parva* *N* = 7, *L. parva* *N* = 16. Predicted values for the full model are based on the estimates listed in Table 2B. Predicted values for the extrinsic model only are based on the following estimates: intercept = 1.57, additive effect = −1.21 environmental effect = 0.57, additive × environment = 0.93.

($\chi^2_1 = 18.44$, $P < 0.0001$). F2 crosses and back-crosses with *L. goodei* animals were intermediate in hatching success between *L. goodei* and *L. parva* conspecific crosses. F2s suffered a 45% reduction in hatching success relative to F1s ($\chi^2_1 = 8.34$, $P = 0.0039$).

The differences between cross types in the low salinity treatment were smaller. Both *L. goodei* and *L. parva* conspecific crosses, F1 crosses, and back-crosses to *L. parva* had high hatching success (>92%). F2 crosses and back-crosses to *L. goodei* had slightly lower hatching success (67–78%). Also, F2s had 16% lower hatching success than F1 animals ($\chi^2_1 = 9.43$, $P = 0.0021$).

Both extrinsic and intrinsic isolation contributed to the reduced hatching success in the F2s and back-crosses to *L. goodei*. Table 2B shows the best-fit model for the line cross analysis. Extrinsic isolation was strongly supported by the large interaction between additive genetic and environmental effects. Intrinsic isolation was also indicated by the presence of dominance–dominance epistasis ($J_{\delta\delta}$). None of the interactions between the various forms of epistasis and environment were included in the best-fit model. Figure 2 shows that the extrinsic isolation model alone overestimates the fitness of the F2s in both the high and low salinity environments. The extrinsic isolation model alone also overestimates the fitness of the back-crosses in the low salinity environment, but matches the observed means well in the high salinity environment. A model that includes both extrinsic isolation plus the effect of dominance–dominance epistasis comes closer to predicting the distribution of the data (Fig. 2B). The effects of dominance–dominance epistasis are consistent with intrinsic isolation because it partially accounts for the lowered fitness of the F2s in both environments (Fig. 2B).

PROPORTION EATING

The proportion of fry surviving to the eating stage was affected by cross and by the interaction between cross and salinity, but not by the overall effects of salinity (Table 1C, Fig. 3). Under low salinity, there were few differences among cross types with the exception of the F2s. F2 crosses had lower survival to the eating stage than all other crosses types. These differences were statistically significant ($P < 0.02$) for all comparisons with the exception of the comparison with back-crosses to *L. parva* ($\chi^2_1 = 3.32$, $P = 0.0684$). Under high salinity, *L. goodei* fry had significantly lower survival to the eating stage than all other cross types ($P < 0.05$). Back-crosses to *L. goodei* had lower survival to the eating stage than both *L. parva* and back-crosses to *L. parva* ($\chi^2_1 = 14.13$, $P = 0.0002$; $\chi^2_1 = 5.93$, $P = 0.0149$). F1 and F2 survival to the eating stage did not differ in the high salinity treatment.

As with hatching success, both extrinsic and intrinsic isolation were indicated in the line cross analysis (Table 2C). Both the effects of additive genetic × environment and dominance–dominance epistasis ($J_{\delta\delta}$) were included in the best-fit model and had sizable effects on the distribution of the data. A model for extrinsic isolation alone overestimates the fitness of the F2s in the low salinity environment, and the fitness of *L. goodei* and F1 and F2 crosses in the high salinity environment (Fig. 3). A model that includes both extrinsic isolation and dominance–dominance epistasis does slightly better at predicting the fitness of the F2s in the low salinity environment and also comes closer to predicting *L. goodei* and F1 fitness in the high salinity environment. However, including $J_{\delta\delta}$ does not increase the ability to predict F2 fitness in high salinity.

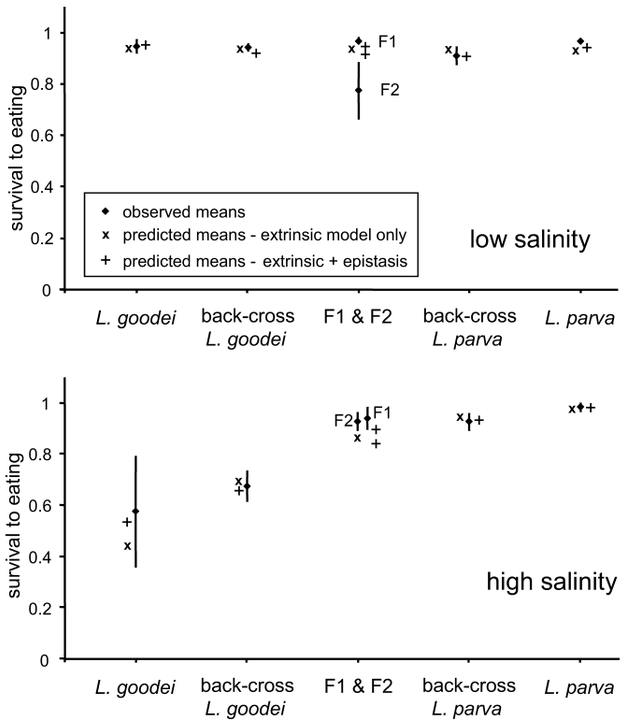

Figure 3. Survival to the eating stage in low and high salinity environments as a function of cross. Means and standard errors are shown. Sample sizes are as follows: low salinity—*L. goodei* $N = 17$, back-cross to *L. goodei* $N = 7$, F1 $N = 13$, F2 $N = 8$, back-cross to *L. parva* $N = 8$, *L. parva* $N = 18$; high salinity—*L. goodei* $N = 3$, back-cross to *L. goodei* $N = 3$, F1 $N = 5$, F2 $N = 4$, back-cross to *L. parva* $N = 7$, *L. parva* $N = 16$. Predicted values for the full model are based on the estimates listed in Table 2C. Predicted values for the extrinsic model only are based on the following estimates: intercept = 2.30, additive effect = −1.05, environmental effect = 0.39, additive × environment effect = 1.09.

Discussion

This study provided evidence for two different patterns in reproductive isolation. The reproductive isolation that emerged as

a function of early egg survival was intrinsic and resulted from epistasis and dominance. In contrast, the reproductive isolation that emerged as a function of hatching success and survival to the eating stage was consistent with both extrinsic and intrinsic isolation. All forms of reproductive isolation were asymmetric. Below, I discuss possible mechanisms for the observed patterns in isolation and their implications for both introgression and divergence in *Lucania*.

The striking pattern for early egg survival was that all crosses involving hybrid G males had an approximate 50% reduction in egg survival. This occurred irrespective of the identity of the female. Methodological artifacts cannot account for this because all crosses were performed at the exactly same time using the exactly same methods. Early egg survival included both fertilization success and subsequent survival until day 5. Furthermore, toward the end of the experiment when we realized that some clutches had high early egg mortality, we visually inspected a subset of these eggs and saw that they lacked the outer membrane present in fertilized eggs and that development had not proceeded through any of the early visible stages (i.e., no blastula). This suggests that either the eggs went unfertilized or that something went awry in the very earliest stages of development.

How can we explain the 50% reduction in early egg survival? One possibility is that incompatibilities cause hybrid G males to be partially (∼50%) sterile. In this case, the incompatibilities are actually manifested in the F1 stage. The fact that hybrid G males had reduced early egg survival regardless of the identity of the females supports this idea. Another possibility is that incompatibilities in hybrid G males may cause a negative paternal effect on embryonic development. Regardless of the mechanism, the reproductive isolation emerging from early egg survival was clearly asymmetrical. Asymmetrical reproductive isolation between species is very common. Asymmetrical incompatibilities obeying Haldane's Rule have been documented in *Drosophila* (Turelli and Orr 1995), Lepidoptera (Presgraves 2002), and mosquitoes (Presgraves and Orr 1998). There is also mounting evidence for such asymmetries in fish (Russell 2003; Bolnick and Near 2005; Russell and Magurran 2006; Kitano et al. 2007).

COMBINED EXTRINSIC AND INTRINSIC ISOLATION

The data for hatching success and survival to the feeding stage provided evidence for extrinsic isolation and genetic incompatibilities in the F2 stage, but no evidence for environmentally dependent genetic incompatibilities. Evidence for extrinsic isolation stems from (1) the statistically significant interaction between environment and additive genetic effects in the line cross analysis and (2) the fact that under the high salinity treatment, *L. parva* had high fitness, *L. goodei* had low fitness, and F2 crosses and back-crosses involving *L. goodei* had intermediate fitness. Hence, crosses that carry a higher proportion of *L. goodei* genes have low survival under high salinity conditions that might act as a barrier to gene flow and increases reproductive isolation.

However, extrinsic isolation cannot completely prevent introgression because the F1 crosses and back-crosses to *L. parva* had high fitness. Any cross that received a complete set of *L. parva* genes had high hatching success under high salinity conditions. Hence, if an *L. goodei* mated with an *L. parva*, then the resulting F1 offspring would have high hatching success. If the F1 mated with an adult *L. parva* (which is likely to be numerically dominant in high salinity environments), then the resulting offspring would also have high hatching success. Rundle (2002) showed a similar pattern in the three-spined stickleback, *Gasterosteus aculeatus*. In that experiment, growth rates were compared between various crosses (benthic, limnetic, and back-crosses to benthics and limnetics) where animals were raised in either the benthic habitat (littoral zone) or in the limnetic habitat (open water). The prediction was that animals with a larger proportion of benthic genes would have high growth rates in the benthic habitat (benthic > back-cross to benthic > back-cross to limnetic > limnetic). Instead, Rundle (2002) showed that benthics and back-crosses to benthics had nearly identical growth rates, but that these growth rates were higher than those for back-crosses to limnetics and limnetic animals (benthic = back-cross to benthic > back-cross to limnetic = limnetic). The pattern was similar to that shown here where a complete set of alleles from one parent in the back-crosses precluded one of the two parent species from having significantly higher fitness than all other cross types in its native environment.

The overall reduced fitness in F2 hybrids provided support for intrinsic isolation. Both the F2 crosses and the back-crosses to *L. goodei* had much lower hatching success than F1 crosses under both salinity treatments, and had lower survival to the eating stage in the low salinity treatment. Including the dominance–dominance epistatic factor in the line cross analysis resulted in predicted means that varied between F1s and F2s that more closely resembled the observed data. However, for back-crosses to *L. goodei,* the inclusion of the dominance–dominance epistatic factor only improved the accuracy of the predicted means for hatching success in the low salinity treatment. Hence, the reduction in fitness for the F2 crosses provided support for intrinsic isolation, whereas the results from the back-crosses to *L. goodei* were more equivocal.

F2 breakdown is generally attributable to the fact that F1s are heterozygous at all loci (except on the XY sex chromosomes of males) and possess a complete set of alleles from each species (Bateson 1909; Muller 1942; Dobzhansky 1951). F2s can be homozygous for either *L. goodei* or *L. parva* alleles at many different loci, and some of these alleles may not function properly together. In organisms other than fish, there is a wealth of empirical studies demonstrating high fitness in the F1 stage, but reduced fitness in the F2 stage (e.g., Burton 1986, 1990; Fishman and Willis

2001; Burton et al. 2006; Demuth and Wade 2007a; reviewed in Endler 1977). Fewer examples exist for fish (but see Russell 2003; Russell and Magurran 2006; Tech 2006; Vigueira et al. 2008) due in part to the increased generation time in fish and the difficulty of rearing animals to the F2 stage.

Despite the fact that the difference in fitness between F1s and F2s appeared to differ between environments—particularly for survival to the eating stage—none of the interactions between environment and epistasis nor between dominance and epistasis were retained in the best-fit models for hatching success or for survival to eating. The interaction between environment and dominance–dominance epistasis was retained in the model for early egg survival but did not have an effect significantly different from zero. The lack of an interaction between environment and epistasis is surprising given the different challenges posed by high and low salinity environments. In low salinity, animals must retain salt, but extricate water. In high salinity, animals must do the opposite and retain water but extricate salt (Evans et al. 2005). One can easily imagine a scenario in which there are gene pathways critical to osmoregulation under high salinity that need not function under low salinity and vice versa for osmoregulation under low salinity, and that different sets of genetic incompatibilities would reduce F2 fitness under high and low salinity conditions. However, this scenario was not supported.

As they stand, the results indicate that intrinsic genetic incompatibilities are expressed independently of environmental conditions. However, two other possibilities may also occur. First, the line cross analysis used in this study only considered two-way epistatic interactions (i.e., interactions between two loci). However, higher order interactions involving many more loci may exist, particularly for physiologically complex traits. The exclusion of higher order interactions (and their interactions with environment) may hamper the ability to detect environmentally dependent genetic incompatibilities. Also, the exclusion of higher level interactions may explain the inability of the line cross analysis to fully predict the reduced hatching success in the F2 stage (Lynch and Walsh 1998; Demuth and Wade 2007a). A second possibility is that environmentally dependent genetic incompatibilities do exist, but that they produce similar net effects, particularly in the reduction of fitness in the F2s. Hence, different incompatibilities occur in different habitats, but reduce fitness by a similar magnitude. QTL studies would be capable of discerning among these three scenarios. However, at present, the most parsimonious explanation is that intrinsic genetic incompatibilities are expressed independently of environmental conditions.

ASYMMETRICAL REPRODUCTIVE ISOLATION

Reproductive isolation was asymmetric between the two species for all three life-history variables, and in all three cases, the direction of the asymmetry suggested that *L. goodei* could more easily introgress into *L. parva* than vice versa. For early egg survival, *L. goodei* females should more easily introgress with *L. parva* because, both their F1 male and female offspring (hybrid P) are viable and fertile. In a population in which *L. parva* is numerically dominant, the resulting F1 offspring would be likely to form back-crosses with *L. parva* which would also have high fitness regardless of their sex. In contrast, *L. parva* females should have a reduced probability of introgressing with *L. goodei* because their F1 sons (hybrid G) will have severely reduced early egg survival. For hatching success and survival until the eating stage, both extrinsic and intrinsic isolation occurred such that *L. goodei* could more easily introgress into *L. parva*, and this was particularly so for the high salinity treatment.

There is some evidence that hybridization in nature is asymmetrical and that *L. goodei* can more easily introgress into *L. parva*. Using meristic characters, Hubbs et al. (1943) surveyed several populations for hybrids between *L. parva* and *L. goodei*. Hybrids were found only in populations where *L. goodei* was $< 5\%$ of the total *Lucania* population (and *L. parva* was $> 95\%$). The assumption was that *L. goodei* females were mating with *L. parva* males. If so, then hybridization in nature occurs in the direction predicted by this study. A similar scenario occurs in the fish genus *Cyprinodon*. Tech (2006) studied a pair of species in which one (*Cyprinodon elegans*) is freshwater and the other (*C. variegatus*) is euryhaline, and found that back-crosses to the euryhaline species were largely successful, whereas back-crosses to the freshwater species produced few juveniles. This suggests a similar asymmetry in reproductive isolation where a freshwater species can introgress into a species that is euryhaline, but the reverse introgression is unlikely. In contrast, studies of hybridization between ecologically similar freshwater fundulid species (*Fundulus notatus*, *F. euryzonus*, and *F. olivaceous*) have not found such effects (Duvernell et al. 2007; Vigueira et al. 2008).

Given the asymmetric reproductive isolation between the two species, why are not hybrids more common in nature? First, prezygotic isolation is present where each species prefers to mate with conspecifics (Fuller et al. 2007). The order in which prezygotic and postzygotic isolation arose and the extent to which prezygotic isolation arose as a consequence of adaptation to salinity are unknown. Another possibility is that asymmetric RI exists at other life-history stages that prevents *L. goodei* from introgressing into *L. parva*. Fuller and colleagues (2007) showed that *L. parva* has overwinter survival roughly 40% that of *L. goodei* in freshwater, but that there are no differences in overwinter survival in brackish water (8 ppt). Unfortunately, this study only considered survival through the larval stage. The extent to which reproductive isolation occurs at this stage and the extent to which this a function of salinity is unknown.

Finally, this study clearly supports the idea that adaptation as a function of salinity can create extrinsic isolation. The

evolutionary transition between freshwater and euryhaline habitats has occurred many times in the family Fundulidae and also in the larger order Cyprinodontiformes to which *Lucania* belong (Wiley 1986; Bernardi 1997). Nearly all freshwater species have decreased egg hatching success above 10 ppt, whereas euryhaline species are much more tolerant of a wide range of salinities (Griffith 1974; Fuller et al. 2007; Fuller 2008). Some euryhaline species have lower overwinter survival to adulthood in freshwater, but these data are available for only a small subset of taxa (Trexler et al. 1990, 1992; Fuller et al. 2007). Regardless, the implication is that salinity is a significant environmental factor in adaptation in fish. The critical task is trying to understand whether extrinsic or intrinsic isolation arose first. Because *L. goodei* and *L. parva* have already diverged, this study provides no information on this subject. However, *L. parva* populations occur across a wide range of salinities (0–70 ppt), and there is museum data indicating that some populations have occurred in relatively constant salinities for at least 50 years (Fuller and Noa 2008; see Hendry et al. 2007 for a review on the speed of ecological speciation). This system would provide fodder for investigations into the evolution of reproductive isolation at a much earlier point in divergence.

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