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Speciation in killifish and the role of salt tolerance

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Abstract

Species pairs whose distributions are tied to environmental conditions provide intriguing candidates for the study of ecological speciation. Here, we examine the role that adaptation to salinity has played in the divergence between two closely related species, *Lucania goodei* and *Lucania parva*, whose distributions reflect salinity (*L. goodei* – fresh water, *L. parva* – euryhaline). We first tested whether these two species display local adaptation and, subsequently, tested for ecological, genic and behavioural isolation by performing crosses within and between *L. goodei* and *L. parva* and raising offspring under various salinities. We found strong evidence for differential adaptation to salinity and also for behavioural isolation where animals preferentially mated with conspecifics over heterospecifics. However, we found no evidence for F1 hybrid inviability. We discuss the general lack of evidence for genic isolation in teleost fish and whether this is a real phenomenon or simply a reflection of experimental design.

Introduction

Over the past decade there has been renewed interest in the idea that divergent natural selection on different populations in different niches leads to reproductive isolation (reviewed in Schluter, 2001; Coyne & Orr, 2004; Rundle & Nosil, 2005; Funk *et al.*, 2006). This model of speciation emphasizes the importance of ecologically based selection to the evolution of reproductive isolation, and has been termed 'ecological speciation'. By contrast, other models have emphasized the role of nonadaptive processes in speciation (e.g. genetic drift, polyploidization and hybridization; reviewed in Coyne & Orr, 2004). In earlier discussions, ecological speciation referred to divergent selection that arises as a segment of the population moves into an empty trophic niche (Schluter, 1996), and parapatric speciation referred to the broader concept of differential adaptation in response to any environmental differences leading to isolation (Endler, 1977; Futuyma, 1986). However, in recent years the term 'ecological speciation' has been used to refer to isolation that arises due to any environmental differences in either abiotic (e.g. temperature, pH and salinity) or biotic (e.g. competitors and predators) factors (Hendry *et al.*, 2000; Schluter, 2001), and we use this definition in this paper.

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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 (Schluter, 2000; Funk *et al.*, 2006). Ecological speciation relies on divergence among populations as a result of adaptation to different environmental conditions and the build-up of reproductive isolation (either premating, gametic or post-zygotic isolation) between the two populations (reviewed in Schluter, 2001; Coyne & Orr, 2004; Rundle & Nosil, 2005). Populations may also become reproductively isolated due to the build-up of genetic incompatibilities that are independent of environmental conditions (i.e. genic isolation) (Coyne & Orr, 2004). Genic isolation is marked by the reduced fitness of hybrids irrespective of environment (Coyne, 1992; Coyne & Orr, 2004).

Ecological and genic isolation can involve any stage of the life cycle (prezygotic, gametic or post-zygotic isolation). Prezygotic, behavioural isolation can result from ecological divergence when there is direct selection on critical mating traits themselves (e.g. traits involved in

pollination – Schemske & Bradshaw, 1999) or when traits evolved in ecological divergence are also important in mating dynamics (Funk, 1998; McKinnon *et al.*, 2004). Alternatively, behavioural isolation can evolve due to differential sexual selection in different populations (Lande, 1981). This may or may not be dependent on ecological conditions (Endler, 1992; Schluter & Price, 1993). Finally, behavioural isolation may evolve due to reinforcement when there is selection to avoid hybrid matings in areas of sympatry (for reviews see Noor, 1999; Servedio & Noor, 2003; Coyne & Orr, 2004). This process can be fuelled by reduced hybrid fitness as a consequence of either ecological or genic isolation. Clearly, the challenge for studies of speciation is to measure reproductive isolation at multiple life stages and to determine the extent to which this is dependent on ecological conditions (e.g. Ramsey *et al.*, 2003; Nosil, 2007). In this paper, we test for the presence of multiple forms of isolation between two killifish species whose distributions differ along a salinity gradient.

The ecological transition from fresh water to sea water that occurs along sea coasts poses a variety of challenges to organisms and is marked by a rapid shift in communities in nearly all groups of aquatic organisms (Gunter, 1945, 1950a, b; Davis, 1955; Godfrey & Wooten, 1979, 1981). Among teleost fish, there is a clear transition from fresh water (0 ppt, parts per thousand, salt) to brackish water (intermediate salinity) to marine (32–35 ppt salt) communities (Gunter, 1945; Boschung *et al.*, 1992; Smith & Bermingham, 2005). This shift in species abundance reflects the differing abilities of species to deal with the different osmotic demands posed by fresh vs. 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 of osmoregulation in the teleost gill see Evans *et al.*, 2005). There are entire groups of fish that are limited to fresh water (e.g. Cypriniformes and Characiformes), and others that are limited to marine environments (e.g. Acanthuridae and Labridae) (Nelson, 2006) suggesting that the evolutionary transition between fresh and salt water is difficult in some groups (Lee & Bell, 1999). Yet other groups contain freshwater, brackish and marine species (e.g. Gasterosteiformes, Fundulidae and Atherinopsidae) with closely related species differing primarily in habitat salinity. For example, in the three-spined stickleback, *Gasterosteus aculeatus*, marine forms have repeatedly invaded freshwater habitats and diverged into benthic and limnetic forms (McPhail, 1984, 1992; Foster *et al.*, 1998; Rundle *et al.*, 2000; see Schluter, 2000, 2001; McKinnon & Rundle, 2002 for reviews). Although the majority of work has focused on divergence between benthic and limnetic forms, divergence has also occurred between anadromous and freshwater populations, resulting in population differences in body size, body shape, foraging traits, armor (body plates), nuptial colouration and courtship most likely due to differences in food types and predators between marine and freshwater habitats (McKinnon & Rundle, 2002). These patterns indicate an important role of salinity in the diversification of teleost fish. Yet, the role of ecological speciation in response to variation in salinity is largely unknown.

In this paper, we test for ecological isolation between two killifish species, *Lucania goodei* and *Lucania parva*, that are found predominantly in fresh and brackish water respectively. We address the following four questions: (1) Is there evidence for adaptation to salinity in *L. goodei* and *L. parva* where *L. goodei* survives best in fresh water and *L. parva* survives best in brackish water? (2) Is there evidence for behavioural isolation between *L. goodei* and *L. parva*? (3) Is there evidence for overall hybrid inferiority (genic isolation) or differential hybrid inviability with respect to salinity (ecological isolation)? (4) Is there evidence for reinforcement (i.e. increased prezygotic isolation in sympatry)? To answer these questions, we performed a series of crosses within and between *L. goodei* and *L. parva* using animals from both sympatric and allopatric populations and raised the offspring under various salinity regimes.

Study system

Genus *Lucania* contains three species belonging to the family of North American killifish, Fundulidae. They were assigned to the genus *Lucania* on the basis of shared morphological characters (Hubbs & Miller, 1965). *Lucania goodei* is found primarily in Florida and coastal Georgia and South Carolina (Lee *et al.*, 1980). *Lucania parva* is found from Cape Cod around the Florida peninsula and to the Gulf of Mexico coast (Lee *et al.*, 1980). The third species, *Lucania interioralis*, is endemic to the Yucatan peninsula of Mexico along the Yucatan coast (Hubbs, 1936). Although there has been no modern phylogenetic work testing the relationships within *Lucania*, allozyme data suggest a genetic distance (*D*) of 0.16 between *L. goodei* and *L. parva* which is consistent with the two species being closely related (Duggins *et al.*, 1983).

Lucania goodei is found primarily in fresh water, whereas *L. parva* is euryhaline and can be found in fresh, brackish and marine habitats. In a review of over 1400 museum records from the University of Florida, R.C. Fuller and L. Noa (unpublished data) found that 93% of *L. goodei* populations were classified as fresh water with the remaining classified as brackish. *Lucania parva* was found readily in all three habitat types with 23% of populations in fresh water, 46% in brackish water and 31% in marine water. These data were approximate because the records simply classified sites as fresh, brackish or marine, and did not provide exact salinity values.

Although the majority of *L. goodei* and *L. parva* populations are allopatric, the two species do co-occur at some sites (Homosassa Springs: Herald & Strickland,

1949; Foster, 1967; Silver Springs: Hubbs & Allan, 1943; Everglades sites: Loftus & Kushlan, 1987). In reviewing museum records from Florida, R.C. Fuller and L. Noa (unpublished data) found that 15% of *L. goodei* populations were sympatric with *L. parva*, and 17% of *L. parva* populations were sympatric with *L. goodei*. Furthermore, there is evidence that hybrids do occur. Hubbs *et al.* (1943) report hybrid fish that were intermediate in meristic characters between *L. goodei* and *L. parva*. Hybrids were < 1% of the combined *L. goodei* and *L. parva* population in sites where hybrids were present. In addition, hybrids were only found in sites where *L. parva* was common (> 90%) and *L. goodei* was rare (< 10%).

Lucania goodei and *L. parva* differ in morphology and colouration (Fig. 1a,b) (Page & Burr, 1991). *Lucania goodei* is longer and more slender than *L. parva*. *Lucania goodei* possess a broad stripe that runs the length of its body which is absent in *L. parva*. Male *L. goodei* are more brightly coloured than male *L. parva*. The anterior section of the dorsal fin is blue in *L. goodei*, but is colourless in *L. parva*. The anal fin of *L. goodei* can be blue, red, yellow, orange or a combination of blue with red, yellow or orange (Fuller, 2002; Fuller & Travis, 2004). *Lucania parva* males occasionally have dull colouration to their anal fins which appears reddish although a systematic study of colouration in this species has not been performed.

The courtship behaviour of *L. goodei* is well documented (Foster, 1967; Fuller, 2001; McGhee *et al.*, 2007). Males interact aggressively with one another establishing territories over patches of vegetation. When a female approaches a male, the male swims in circles around the female and flicks its head either in front or laterally to the female. The extent of circle swimming can be quite variable (K.E. McGhee personal communication). If the female remains on the territory, then the male takes a position beneath the female where the male flicks its head and rubs the underside of the female. The two

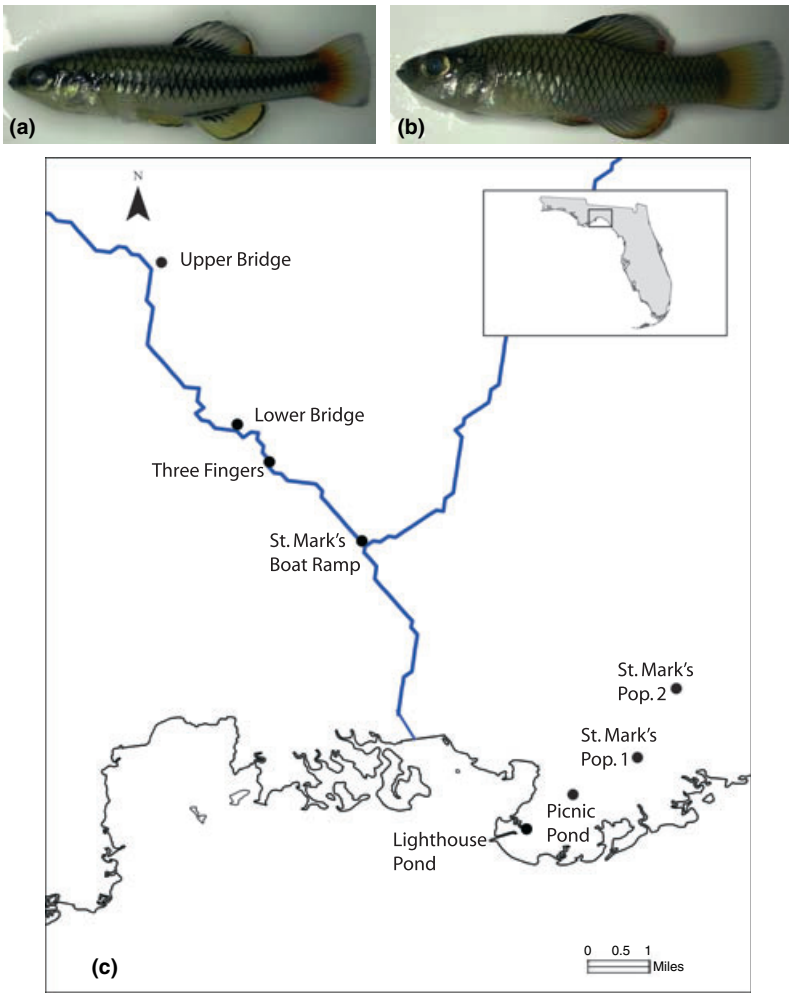

Fig. 1 (a) Male *Lucania goodei* and (b) male *Lucania parva*. (c) A map of the sites.

animals then swim together and press their bodies onto the vegetation releasing egg and sperm. Foster (1967) describes the spawning behaviour of *L. parva* as being similar to that of *L. goodei* with the exception that males of *L. parva* do not perform head flicks in the early stages of courtship (i.e. in front or laterally to the female), but do perform head flicks once they have taken a position beneath the female in the latter stages of courtship. However, Foster (1967) observed fish from only one locality, and the sample sizes were not reported. Hence, the variation in *L. parva* courtship behaviour is unknown.

Methods

Field survey

We collected *L. goodei* and *L. parva* at eight sites in the Wakulla–St Mark's River drainage in Wakulla County, FL, during May 2005. We collected animals with dipnets and seines and recorded salinity in ppt with a YSI-63 meter (YSI Inc., Yellow Springs, OH, USA). Our goal was to collect a minimum of 60 individuals of either *L. goodei* or *L. parva*, although this was not always possible (see Table 1). From these data, we determined the relative abundance of each species and its relation with salinity.

We continued to monitor four sites: Upper Bridge, Lower Bridge, Three-Fingers and Lighthouse Pond. We censused these sites and recorded the salinity in October 2005, January 2006, March 2006, August 2006 and January 2007.

Breeding study – 2005

There were two goals for our breeding study: (1) to generate eggs to examine survival at various salinities and life-history stages for *L. goodei*, *L. parva* and their F1 hybrids; and (2) to measure premating isolation between *L. goodei* and *L. parva* in both sympatric and allopatric populations. To do this, we collected *L. goodei* and *L. parva* from allopatric and sympatric populations. We collected *L. goodei* from the Upper Bridge and Three-Fingers populations and *L. parva* from Three-Fingers and Lighthouse Pond sites. We brought the animals to the laboratory at Florida State University and maintained them in stock aquaria at 2 ppt salinity. We fed them one to two times each day with frozen *Artemia* and bloodworms. The light ratio was 14L : 10D, and the room temperature was at 21 °C.

We performed both conspecific and heterospecific crosses within and between *L. goodei* and *L. parva* using animals from both allopatric and sympatric populations. Specifically, our experiment had two treatments. 'Allopatry vs. Sympatry' refers to whether animals came from allopatric or sympatric populations. For allopatric crosses, we used *L. goodei* from the Upper Bridge population and *L. parva* from the Lighthouse Pond population. For sympatric crosses, both *L. goodei* and *L. parva* came from the Three-Fingers population. The Upper Bridge population has been allopatric for *L. goodei* since at least 1950 (University of Florida Museum Record no. RWY155). Over the past 55 years, there has been no record of *L. parva* at this site (R.C. Fuller and L. Noa, unpublished data; R.C. Fuller personal observation; J. Travis, personal communication). Similarly, Lighthouse Pond has been allopatric for *L. parva* since at least 1954 (Record no. ZDKC189). This site is a long-term study for *Poecilia latipinna*, and *L. goodei* has not been recorded there (R.C. Fuller and L. Noa, unpublished data, J. Travis, personal communication). The sympatric Three-Fingers site was found in May 2005 (RCF and KEM). Subsequent examinations of museum records from the University of Florida indicate that this site was sampled in 1975 and that it was sympatric. However, there are no records between 1975 and 2005. Fig. 1c shows the location and distance between these populations in north Florida.

'Cross' refers to the manner in which the two species were paired. We had four crosses: (1) *L. goodei* ♀ × *L. goodei* ♂, (2) *L. goodei* ♀ × *L. parva* ♂, (3) *L. parva* ♀ × *L. goodei* ♂, (4) *L. parva* ♀ × *L. parva* ♂. This results in eight experimental treatments. We performed three replicates of each treatment resulting in 24 pairs of fish. One tank that was supposed to be a sympatric cross of *L. goodei* ♀ × *L. goodei* ♂ was accidentally labelled as a sympatric *L. parva* ♀ × *L. goodei* ♂. Hence, there were only two replicates of sympatric *L. goodei* ♀ × *L. goodei* ♂ and four replicates of sympatric *L. parva* ♀ × *L. goodei* ♂.

Table 1 Salinity, total abundance and relative frequencies of *Lucania goodei* and *Lucania parva* across eight populations in May 2005.

Population	Salinity (ppt)	No. of *L. goodei*	No. of *L. parva*	Frequency of *L. goodei*	Frequency of *L. parva*
1. Upper Bridge	0.1	200	0	1	0
2. St Mark's Population 2	0.1	59	0	1	0
3. Lower Bridge	0.2	157	6	0.963	0.037
4. Three-Fingers	0.2	44	53	0.454	0.546
5. Picnic Pond	0.8	14	0	1	0
6. St Mark's Boat Ramp	1.3	7	36	0.163	0.837
7. Lighthouse Pond	5	0	100	0	1
8. St Mark's Population 1	3	0	5	0	1

For each cross, we placed one female and one male in a 19-L aquarium containing water at 2 ppt salinity. Each aquarium had four spawning mops which served as spawning substrate. Two mops had floats and two had sinkers, providing spawning substrate both at the bottom of the tank and slightly below the water surface. We checked mops once every 2–3 days for eggs from 10 June to 20 July 2005. From these data, we calculated the total number of eggs produced and the latency until the first spawn. Latency until first spawn was equivalent to the day when eggs were first found in the spawning mops. We denoted 10 June as day 0 and 20 July as day 40. Pairs that failed to spawn were conservatively assigned a latency of 40 days. We used the latency to spawn and the total number of eggs spawned as measures of behavioural isolation.

A few adult fish died in this experiment (three females and two males). Most deaths occurred because animals were hiding in mops and were accidentally dropped on the floor during egg checks. These animals were replaced with animals from the stock tanks. In all five cases, the spawning status of the pair did not change after introduction of a new individual. In four cases, the pairs had spawned prior to the death of one fish, and the new pairs (with the replacement fish) also spawned. In one case, neither the original pair nor the new pair spawned.

To examine the effect of salinity on survival in early life stages, we raised eggs under 0, 2, 4 or 8 ppt salinity. We chose this range in salinity because the results of our field census indicated that the transition between allopatric *L. goodei* and allopatric *L. parva* populations occurred between 0.2 and 2 ppt salinity (see *Results*). Our goal was to raise 10 eggs from each pair of spawning fish in each of the four salinity treatments. For the 0-ppt treatment, we used pure well water which is naturally high in dissolved minerals. For the 2-, 4- and 8-ppt salinity treatments, we added Instant Ocean Sea Salts© to well water. We recorded the proportion of eggs that hatched and the proportion of fry that made the transition to independent feeding. Previous work shows that these are critical transitions in early growth and survival (McCune *et al.*, 2002). To avoid order effects, we assigned the eggs to salinity treatments in random order. During the experiment, it became clear that some hybrid crosses were producing exceedingly few eggs. Because we also wanted to compare survival between conspecific and heterospecific crosses, we first subjected these eggs to 2 ppt and then followed a random order thereafter. The first batches of eggs were assigned to the 2-ppt treatment. Subsequent batches of eggs were assigned to 0, 4, and 8 ppt treatments at random.

Breeding study – 2006

Preliminary analyses of the first breeding experiment had low power for some tests, so we repeated the breeding experiment in 2006 and more than doubled the sampled sample size. We used the same methods except where indicated below.

We again collected animals from both allopatric and sympatric sites in March 2006. Unfortunately, Hurricane Dennis significantly altered the Three-Fingers population in July 2005. As a result of high storm surge, the Three-Fingers population experienced full strength sea water for about 48 h. Both *L. goodei* and *L. parva* were present at this site following the hurricane, but the aquatic vegetation died and degraded over the next several months. In October 2005 and January 2006, both species were present in low abundance (Table 2). In March 2006, *L. parva* was again present in high abundance, but *L. goodei* was absent (Table 2). Hence, we were forced to use the Lower Bridge site as our sympatric population (see Fig. 1c for map). Subsequently, in August 2006 and January 2007, both species were present at the Three-Fingers population.

For *L. goodei*, we collected animals from the Upper Bridge (allopatric) and Lower Bridge populations (sympatric). For *L. parva*, we collected animals from the Lighthouse Pond (allopatric) and Lower Bridge populations (sympatric). Historically, the Lower Bridge population has been allopatric for *L. goodei* (R.C. Fuller & L. Noa, unpublished data). University of Florida records indicate that this site was allopatric for *L. goodei* from 1950 to 1971. J. Travis (personal communication) sampled this site periodically since 1981 and found only *L. goodei*. In May 2005, we found that this population was now sympatric for *L. goodei* and *L. parva*. Subsequent samples at this population indicate that it has remained sympatric for the two species (Table 2). We assume that the *L. parva* are derived from sympatric populations downstream.

Table 2 Census data from October 2005 to January 2007.

Site	Month	No. of *L. goodei*	No. of *L. parva*	Salinity
Upper Bridge	October 2005	60	0	0.1
	January 2006	60	0	0.1
	March 2006	74	0	0.1
	August 2006	60	1	0.1
	January 2007	70	0	0.1
Lower Bridge	October 2005	12	60	0.2
	January 2006	58	10	0.2
	March 2006	44	56	0.2
	August 2006	22	60	0.2
	January 2007	68	13	0.2
Three-Fingers	October 2005	3	44	0.2
	January 2006	1	6	0.2
	March 2006	0	21	0.2
	August 2006	33	37	0.2
	January 2007	1	69	0.2
Lighthouse Pond	October 2005	0	60	25.8
	January 2006	0	60	23.3
	March 2006	0	60	20.2
	August 2006	0	85	29.8
	January 2007	0	44	20.1

We brought the animals back to the laboratory at the University of Illinois and maintained them in stock aquaria at 2 ppt. Unlike the situation at Florida State University (where we had access to well water which was nearly identical to spring water), we had to use treated water at the University of Illinois. We treated city water with StartRite water conditioner which removes all chlorine (Jungle Laboratories, Cibolo, TX, USA). We then filtered the water to remove ammonia (resulting from chloramine). This resulted in hard, fresh water which is suitable for *Lucania*. All other animal husbandry protocols were identical to those in the previous experiment.

We again performed four types of crosses (*L. goodei* conspecific cross, *L. parva* conspecific cross and hybrid crosses in both directions) using both allopatric and sympatric animals resulting in eight experimental treatments. We performed seven replicates resulting in 56 aquaria each with a pair of fish. Aquaria contained water at 2 ppt salinity, and each aquarium had two mops with floats and two mops with sinkers. We checked mops once every 2–3 days from 24 April 2006 to 2 June 2006. From these data, we calculated the total number of eggs produced and the latency until the first spawn. We denoted 24 April as day 0 and 2 June as day 40. Pairs that failed to spawn were conservatively assigned a latency of 40 days. For tanks that had not produced a minimum of 10 eggs for each of the salinity treatments (but had produced more than zero eggs), we continued to check for eggs for the next 2 months, but these data were not included in the behavioural isolation data. We stopped all mop checks on 5 July 2006.

As in the 2005 experiment, we raised eggs and fry at 0, 2, 4 and 8 ppt salinity. Once we had obtained a minimum of 10 eggs for each of the four salinities, we continued collecting eggs and raised them at 15 and 20 ppt. As before, we measured the proportion of eggs hatching and the proportion of fry living to the eating stage.

Survival to adulthood in stock tanks – 2005

We measured survival to adulthood in *L. goodei*, *L. parva* and both types of hybrids (hybrid A – *L. goodei* ♀ × *L. parva* ♂; hybrid B – *L. parva* ♀ × *L. goodei* ♂) in oval stock tanks at various salinity levels using the juvenile fish generated from the 2005 breeding experiment. We measured survival to adulthood in *L. goodei* and *L. parva* at 0, 2, 4, and 8 ppt salinity (four tanks per treatment for *L. goodei* and three tanks per treatment for *L. parva*). Due to low numbers, we only raised hybrids to adulthood at 2 ppt salinity (four tanks for hybrid A; four tanks for hybrid B). For each tank, we added 10 juvenile fish that were at least 1 month old to each stock tank on 25–26 August 2005. Due to limitations on the number of available stock tanks, we were not able to examine the effect of allopatric vs. sympatric populations. We tried to use animals from a single spawning pair of fish for each stock tank whenever possible, but we had to combine animals from different pairings and also between allopatric and sympatric populations in some cases.

Stock tanks were set up outdoors behind the Mission Road Greenhouse at Florida State University. Each stock tank was filled with well water (568 L), and salinity treatments were created by adding an appropriate amount of Instant Ocean Sea Salt© (aquarium Systems, mentor, OH, USA). Fish were fed *Artemia* nauplii and frozen *Artemia* adults once every 3 days. In addition, the fish had access to naturally occurring food items present in the tanks (e.g. algae and small aquatic invertebrates). Salinity was checked and adjusted once a month. The salinity naturally fluctuated somewhat because of either evaporation (salinity increases) or rainfall (salinity decreases). This variation in salinity was low, and all of the salinity treatments fluctuated in a similar manner. On 22 March 2006, we censused the stock tanks and determined the number of adults and juveniles in each stock tank, as well as their standard lengths (which we refer to as body size).

Statistical analysis

For both breeding experiments, we considered latency to spawn and the total number of eggs spawned as measures of behavioural isolation. Both parameters had a truncated, exponential distribution. We therefore used a maximum-likelihood model that assumed a gamma distribution and a log-link function to examine the effects of cross, allopatry vs. sympatry, year and their interactions. Because we used a log-link function, we added a small constant (one) to all the observations. In addition to accommodating the distribution of the data, a maximum-likelihood approach was deemed necessary due to heteroscedastic variances among treatments. These analyses were robust to multiple statistical approaches (i.e. modelled as a gamma distribution with a log-link function, a generalized linear model on log-transformed variables with a normal distribution, or as a general linear model of log-transformed variables). From these models, we determined the least-squares means and 95% confidence limits on the log scale. To control for the effect of year, we present the back-transformed (anti-log) least squares means and 95% confidence limits.

Next, we analysed the probability of eggs hatching and the probability of fry eating. We used a logit analysis assuming a binomial distribution. We considered only those clutches that contained at least five eggs. For some treatments (i.e. hybrid crosses from a particular population), we only obtained a single clutch. Hence, we were unable to analyse a full model (which would include cross, allopatry vs. sympatry, salinity and all possible interactions). We therefore considered only a model that included the effects of cross, salinity, year and their interactions. We first analysed the probability of hatching and surviving until the eating stage at 0–8 ppt salinity for the 2005 and 2006 data. For presentation, we show the back transformed (i.e. inverse logit) least-squares means

and 95% confidence limits to control for the effect of year (Littell *et al.*, 2002).

We then performed a second analysis where we considered hatching and survival from 0 to 20 ppt salinity for the 2006 data. A significant effect of cross where hybrids have lower hatching success or where hybrids are less likely to make the transition to independent foraging would be consistent with intrinsic post-zygotic isolation (i.e. genic isolation). A significant effect of cross by salinity would be consistent with extrinsic incompatibility.

For the stock tank experiment, we performed two sets of analyses. The first set of analyses examined survival to adulthood and adult size in *L. goodei* and *L. parva* at the various salinities. Specifically, we examined the effects of cross, salinity and their interaction. The second set of analyses compared survival to adulthood and adult size between *L. goodei*, *L. parva*, and the two types of hybrids at 2 ppt salinity. For survival to adulthood, we again used a maximum-likelihood model assuming a binomial distribution with a logit link function. For adult size, the data were normally distributed, and we used a general linear model. For all linear models, we present the results from a type 3 model. To examine pairwise differences within statistically significant main effects, we use Tukey's multiple comparisons tests for an analysis of variance models and least-square means for maximum-likelihood models. All analyses were performed in SAS v. 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

Field survey

As expected, *L. goodei* is more common in fresh water and *L. parva* increases in abundance as salinity increases ($r = 0.788$, $P = 0.02$, $n = 8$). However, contrary to previous reports from Everglades populations (Loftus & Kushlan, 1987), there was a site where *L. goodei* and *L. parva* occurred in roughly equal proportions (Three-Fingers). At sites where the two species co-occurred, we found no obvious microhabitat partitioning. We routinely caught *L. goodei* and *L. parva* in the same seine haul or dipnetted them in the same location.

Table 2 shows the results from our repeated census of four sites. Three important points emerge. First, following Hurricane Dennis (July 2005), the salinity at Lighthouse Pond increased from 5 ppt (Table 1) to > 20 ppt (Table 2). This was because of high storm surge where Lighthouse Pond was under sea water for over 48 h. The salinity values at the other sites were constant across multiple censuses. Second, the Lower Bridge site was always sympatric, and the Three-Fingers site was usually sympatric with the exception of March 2006. Third, we did find a single *L. parva* at the Upper Bridge site in August 2006. This is notable because museum records indicate that this site has been allopatric for *L. goodei* for over 50 years. In other words, this was the first time in over 50 years that *L. parva* had been found at this site. Travis has periodically sampled this site since the late 1980s and has never found *L. parva* (J. Travis, personal communication). Furthermore, R.C. Fuller (unpublished data) has sampled this site multiple times a year since 1998 and has never found *L. parva*. We did not find *L. parva* at the Upper Bridge site in January 2007.

Breeding study

The analysis of total eggs spawned reveals striking behavioural isolation. Table 3 shows the likelihood-ratio statistics for a type 3 analysis. On average, conspecific crosses produced 3.7 times more eggs than hybrid crosses (Fig. 2a). There was also an effect of allopatry vs. sympatry where allopatric animals produced 2.2 times more eggs than sympatric animals. However, there was no evidence to suggest reinforcement as the interaction between allopatry vs. sympatry and cross was not significant.

A similar pattern emerged from the analysis of latency to spawn (Table 4, Fig. 2b). Strong behavioural isolation was indicated as conspecific crosses began spawning almost immediately, whereas hybrid crosses began spawning, on average, 13.2 days later. These results are conservative because pairs of fish that never spawned were assigned a latency of 40 days. There were also statistically significant effects of allopatry vs. sympatry, year and cross by year. On average, animals from the sympatric population began spawning 2 days later than animals from the allopatric populations. Similarly, animals from the 2005 breeding study began spawning 3.5 days earlier than animals from the 2006 breeding study. The interaction between cross and year was attributable to the fact that *L. goodei* began spawning earlier in 2005, whereas *L. parva* began spawning earlier in 2006 (Fig. 2c). Finally, there was a marginally non-significant interaction between cross and allopatry vs. sympatry (Table 4). However, there was no evidence to suggest reinforcement. Neither of the hybrid crosses involving allopatric animals began spawning significantly sooner than crosses involving sympatric animals (*post hoc*

Table 3 Likelihood-ratio statistics for a type 3 analysis for total eggs spawned plus 1 (deviance/d.f. = 1.51).

Source	d.f.	χ^2	*P*
Cross	3	23.41	< 0.001
Allopatry vs. sympatry	1	9.07	0.003
Cross × allopatry vs. sympatry	3	2.44	0.486
Year	1	0.72	0.397
Year × cross	1	2.12	0.548
Year × allopatry vs. sympatry	1	2.65	0.103
Year × cross × allopatry vs. sympatry	3	1.34	0.719

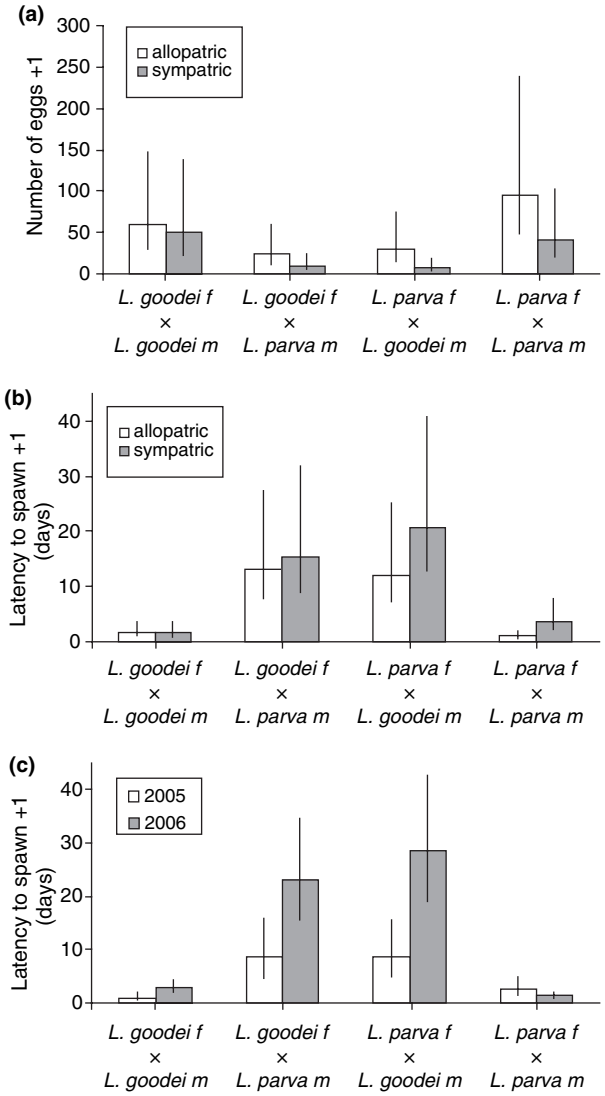

Fig. 2 (a) The number of eggs plus one and (b) the latency to spawn plus one as a function of cross and allopatry vs. sympatry. (c) The latency to spawn as a function of cross and year. Animals that did not spawn were conservatively assigned a latency to spawn of 40 days. Back-transformed least-squares means and 95% confidence limits are shown. f = female. m = male.

Table 4 Likelihood-ratio statistics for a type 3 analysis for latency to spawn +1 (deviance/d.f. = 0.81).

Source	d.f.	χ^2	*P*
cross	3	64.13	< 0.001
Allopatry vs. sympatry	1	6.05	0.014
Cross × allopatry vs. sympatry	3	7.20	0.066
year	1	9.93	0.002
Year × cross	1	15.14	0.002
Year × allopatry vs. sympatry	1	2.54	0.111
Year × cross × allopatry vs. sympatry	3	1.03	0.793

tests: *L. goodei* ♀ × *L. parva* ♂: $\chi_1^2 = 0.16$, $P = 0.685$; *L. parva* ♀ × *L. goodei* ♂: $\chi_1^2 = 2.20$, $P = 0.138$).

Effects of salinity on eggs

There was no evidence of differential egg survival as a function of cross, salinity or their interaction when eggs were reared at 0, 2, 4, and 8 ppt salinity (Table 5). Hatching success was slightly higher in 2005 than in 2006 (0.90 and 0.82 respectively). There was also a statistically significant interaction between cross and year (Fig. 3). In 2005, there were no differences in hatching success between crosses. In 2006, conspecific *L. parva* crosses had higher hatching success than conspecific *L. goodei* crosses and both types of hybrid crosses. There were no statistically significant effects on the probability of fry surviving to the eating stage. Overall, fry survival was very high (0.97).

There was strong evidence for differential survival between crosses at higher salinities (Table 6, Fig. 4). An analysis of the 2006 data (which includes eggs raised at 15 and 20 ppt) indicated a highly significant interaction between cross and salinity (Table 6) due to the fact that hatching success decreased dramatically for *L. goodei* at 15

Table 5 Logit analysis for the probability of eggs hatching in 2005 and 2006.

Source	d.f.	χ^2	*P*
Cross	3	4.13	0.248
Salinity	3	1.11	0.776
Cross × salinity	9	7.65	0.569
Year	1	18.18	< 0.001
Year × cross	3	23.8	< 0.001
Year × salinity	3	2.22	0.528
Year × cross × salinity	9	14.95	0.092

This analysis is for eggs raised in 0, 2, 4 or 8 ppt salinity (deviance/d.f. = 2.2).

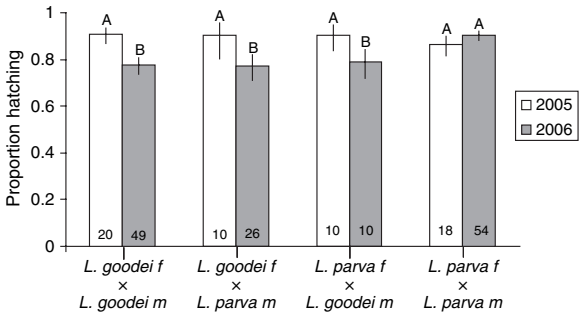

Fig. 3 The proportion of eggs hatching as a function of cross and year. Back-transformed least-squares means and 95% confidence limits are shown. Number of clutches for each treatment is shown at the bottom of the graph. Letters denote statistically significant differences.

Table 6 Logit analysis for the probability of eggs hatching for eggs from the 2006 breeding study.

Source	d.f.	χ^2	*P*
Cross	3	223.55	< 0.001
Salinity	5	9.85	0.0795
Cross × salinity	15	115.34	< 0.001

This analysis includes eggs raised at 0, 2, 4, 8, 15 and 20 ppt salinity (deviance/d.f. = 2.1).

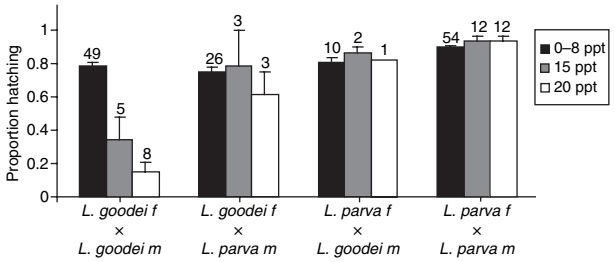

Fig. 4 The proportion of eggs hatching as a function of cross and salinity in 2006. For presentation, hatching success in 0–8 ppt was pooled. Mean ± SE on the original, untransformed data are shown. Number of clutches for each treatment is shown above error bars.

and 20 ppt. Hatching success decreased with increasing salinity in conspecific *L. goodei* crosses ($r_s = -0.388$, $P = 0.002$, $n = 62$), but not in either of the hybrid crosses (*L. goodei* ♀ × *L. parva* ♂, $r_s = 0.207$, $P = 0.256$, $n = 32$; *L. parva* ♀ × *L. goodei* ♂, $r_s = 0.197$, $P = 0.518$, $n = 13$); or in conspecific *L. parva* crosses ($r_s = 0.194$, $P = 0.089$, $n = 78$). Specifically, *L. goodei* hatching success was one-third that of *L. parva* in 15 ppt and one-sixth that of *L. parva* in 20 ppt. An analysis of survival to the eating stage was not possible due to low sample sizes in *L. goodei*. Because of high mortality in the egg stage, there were no clutches with a minimum of five fry in the 15- or 20-ppt salinity treatments to examine subsequent survival.

Survival to adulthood in stock tanks

Salinity had strong, differential effects on survival to adulthood in *L. goodei* and *L. parva* (Table 7a, Fig. 5a). Survival to adulthood increased with salinity in *L. parva* ($r_s = 0.613$, $P = 0.034$, $n = 12$). Survival in *L. goodei* tended to decrease with salinity, although this was not statistically significant ($r_s = -0.317$, $P = 0.231$, $n = 16$). There were also strong, differential effects of salinity on adult body size (Table 7b, Fig. 5b). Adult size decreased with salinity in *L. goodei* ($r_s = -0.679$, $P = 0.004$, $n = 16$) mainly due to *L. goodei* having significantly smaller body size at 8 ppt than at 0, 2 or 4 ppt salinity. *Lucania parva* reached its greatest size when raised at 4 ppt. Adult body size in *L. parva* at 4 ppt was significantly greater than at 0 or 8 ppt.

Because of low numbers of hybrid offspring in 2005, we could only raise hybrids at 2 ppt. F1 hybrids were

Table 7 (a) Logit analysis of the probability of surviving to adulthood (deviance/d.f. = 4.0) (b) analysis of variance on adult size as a function of species, salinity and their interaction (Mean square/MS).

Source	d.f.	χ^2	*P*
(a) Survival to adulthood			
Species	1	13.84	< 0.001
Salinity	3	10.68	0.014
Species × salinity	3	31.07	< 0.001

Source	d.f.	MS	*F*	*P*
(b) Adult body size				
Species	1	109.8	23.3	< 0.001
Salinity	3	57.8	12.3	< 0.001
Species pix salinity	3	20.8	4.4	0.016
Error	19	4.7		

These analyses only consider differences between *Lucania goodei* and *Lucania parva*.

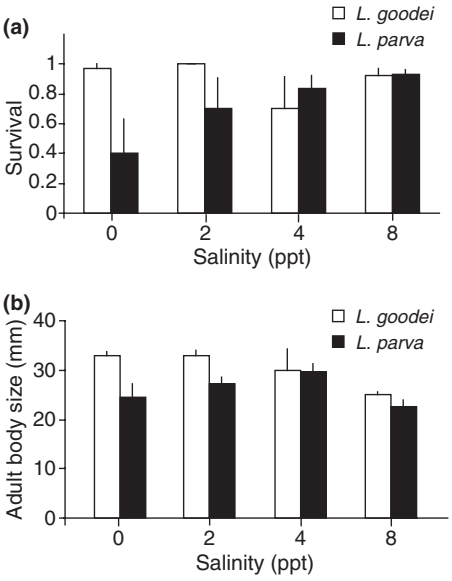

Fig. 5 (a) Survival to adulthood and (b) adult body size in mm for *Lucania goodei* and *Lucania parva* as a function of salinity. Means ± SE calculated on the original, untransformed data are shown. For adult body size at 0 ppt, $n = 2$ for *L. parva*. For all other means, at each salinity, $n = 4$ for *L. goodei* and $n = 3$ for *L. parva*.

intermediate in both survival and adult body size in comparison with *L. goodei* and *L. parva* raised at 2 ppt (Fig. 6, Survival – Cross $\chi_3^2 = 17.9$, $P < 0.001$; Body size – Cross $F_{3,11} = 20.05$, $P < 0.001$). The survival of both hybrids was significantly greater than that of *L. parva* and was significantly lower than *L. goodei* (which was 100%). In terms of body size, *L. parva* was significantly smaller than both hybrids as well as *L. goodei*, although there were no statistically significant differences between *L. goodei* and the hybrids.

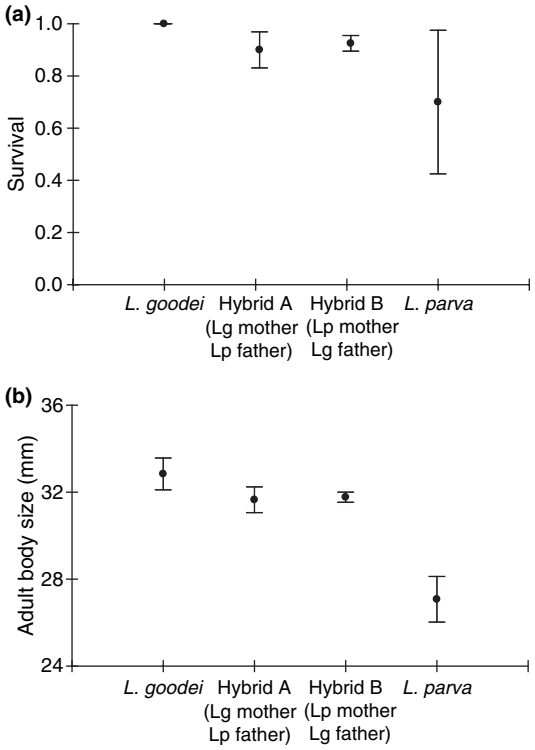

Fig. 6 (a) Survival to adulthood and (b) adult body size in mm for *Lucania goodei*, *Lucania parva* and both hybrids at 2 ppt. Means and standard errors calculated on the original, untransformed data are shown. Means ± SE calculated on the original untransformed data. *L. goodei*: $n = 4$, *L. parva*: $n = 3$, hybrid A: $n = 4$, hybrid B: $n = 4$.

Discussion

The process of ecological speciation consists of three components; an ecologically based source of divergent selection, a mechanism of reproductive isolation, and a genetic link between divergent selection and reproductive isolation (Rundle & Nosil, 2005). In this study, we examined two of these components, an ecological source of divergent selection (salinity) and the importance of three types of reproductive isolation (ecological, behavioural and genic isolation).

Adaptation to salinity

Our results provide evidence for ecological divergence between *L. goodei* and *L. parva*, and the nature of this divergence roughly reflects their distributions across salinity. *Lucania goodei* is more common in fresh water, whereas *L. parva* is more common in brackish water. Based on the results of this field study, we hypothesized that local adaptation to salinity exerts an ecological source of divergent selection on *L. goodei* and *L. parva*. Consistent with this hypothesis, we found differential effects of salinity on survival to adulthood and adult body size when animals were reared outside in stock tanks. *Lucania parva* had reduced survival to adulthood in fresh water (0 ppt), whereas *L. goodei* had reduced growth at 8 ppt and reduced hatching success at 15 and 20 ppt. However, these results do not explain why *L. parva* is frequently found in freshwater populations. In our study, *L. parva* was found in two freshwater sites (Lower Bridge and Three-Fingers). Furthermore, in a review of museum records, R.C. Fuller and L. Noa (unpublished data) found that 23% of *L. parva* populations were in fresh water.

One possible explanation for this discrepancy is that the animals in our stock tanks not only had to survive to adulthood, but they also had to overwinter, and thus, be able to withstand colder temperatures. Work on *Fundulus heteroclitus* suggests that enzymes critical to osmoregulation perform poorly in cold temperatures (Kidder *et al.*, 2006). Similarly, in an experiment investigating the relative effects of salinity, temperature and food, Trexler *et al.* (1990) demonstrated that female sailfin mollies (*P. latipinna*) had the slowest growth when raised in cold temperatures and at low salinities. Similarly, sailfin mollies maintained in field cages usually had higher overwinter survival in salt water compared with fresh water (Trexler *et al.*, 1992). This suggests that an interaction between salinity and temperature may be the critical abiotic factors that differentiate *L. goodei* and *L. parva*. This also potentially explains *L. parva*'s presence in many freshwater springs and spring fed rivers (Homosassa Springs: Herald & Strickland, 1949; Foster, 1967; Silver Springs: Hubbs & Allan, 1943). In addition to having high levels of dissolved ions, freshwater springs are remarkably constant in temperature year round (21 °C). Hence, springs may be tolerable for *L. parva* during winter. The idea that a combination of salinity and temperature are the critical abiotic variables distinguishing *L. goodei* and *L. parva* is also in keeping with early verbal hypotheses on the original divergence between these two taxa. Duggins *et al.* (1983) suggested that these two groups may have diverged in the Pleiocene when one population invaded fresh water during a time of heightened global cooling. Proper phylogenetic work is needed to establish the direction of the invasion (i.e. marine to fresh water vs. fresh water to marine) as well as the timing of the divergence.

The trade-off across salinity for *L. goodei* and *L. parva* is all the more striking when one considers that our experimental design precluded competition between the two species. Earlier work by Dunson & Travis (1991) found evidence for differential effects of salinity on competition between *L. goodei* and *L. parva*. In their experiment, they raised *L. goodei* and *L. parva* either alone or in combination at 0 and 15 ppt salinity and measured survival and growth. *Lucania goodei* grew faster in 0 ppt than in 15 ppt and the difference between 0 and 15 ppt increased when *L. goodei* was raised in competition with *L. parva*. *Lucania parva* also grew faster in 0 ppt than in 15 ppt, but this pattern reversed when raised in compe-

tition with *L. goodei*. The main result was that salinity (0 vs. 15 ppt) altered the effects of competition between *L. goodei* and *L. parva*. Hence, the trade-off in survival across salinity may be even greater in nature than that found in this study.

Mechanisms of reproductive isolation

This study provides strong evidence for the role of behavioural isolating mechanisms in the maintenance of the two species, *L. goodei* and *L. parva*. Heterospecific pairs had a longer latency to spawn and produced fewer eggs than conspecific pairs of either *L. goodei* or *L. parva*. This strong pattern emerged using a relatively conservative one-way choice test where animals were given a choice between spawning (with whomever they were paired) and not spawning at all (see Houde, 1997; Coyne & Orr, 2004 for discussions of one-way choice tests; see Nagel & Schluter, 1998; Boughman, 2001; Boughman *et al.*, 2005 for stickleback experiments using one-way choice tests). In 2005, we isolated gravid females and reproductive males in aquaria at the height of the spawning season. In 2006, we isolated gravid females at the start of the spawning season. We typically have great success in getting animals to spawn at these times. Despite this, many hybrid pairs had a long latency to spawn and some failed to spawn at all indicating strong prezygotic isolation.

The evolutionary mechanism via which behavioural isolation arose between *L. goodei* and *L. parva* is unknown. Given that the two species occur in sympatry in some areas, reinforcement (selection against forming hybrids in areas of sympatry) is a possibility. However, given that we found no evidence for reinforcement, this would require the spread of genes conferring behavioural isolation from sympatric populations to allopatric populations for both species. Another possibility is that sexual selection has occurred independently in the two lineages and resulted in the evolution of different signals and preferences. The critical issue is determining the extent to which ecological divergence between the two species contributed to behavioural isolation. Another critical assumption is that the behavioural isolation between the two species is the result of genetic differentiation. Because this experiment was performed with wild-caught animals, we cannot rule out the possibility that learning (i.e. environmental effects) has also contributed to behavioural isolation.

The actual mechanism that creates prezygotic isolation in *Lucania* is currently unknown. Given that *L. goodei* and *L. parva* differ in body size, male colouration and male courtship, we assume that a critical cue is missing which makes animals reluctant to mate. However, we currently do not know whether prezygotic isolation is a function of male behaviour, female behaviour or both. Another possible scenario is that heterospecifics do mate, but then subsequently cannibalize their eggs. Egg cannibalism is high in *L. goodei* (Fuller & Travis, 2001) and appears to be important in reproductive isolation in other external fertilizers (Albert & Schluter, 2004). Although we cannot rule out this alternative, we find it unlikely. We found no eggs in the spawning substrates of many heterospecific pairs, meaning that they would have had to quickly eat all of their eggs after every spawning.

This work adds to the growing body of literature indicating the importance of behavioural isolation in teleost fish (Endler & Houde, 1995; Seehausen *et al.*, 1997; Rundle & Schluter, 1998; Ishikawa & Mori, 2000; Albert & Schluter, 2004; Alexander & Breden, 2004). In sticklebacks, reproductive isolation has arisen between benthic and limnetic forms irrespective of lakes (i.e. whether the morphs recently shared an ancestor) (Rundle *et al.*, 2000), and this isolation is primarily dependent on differences in body size between the two morphs that have arisen due to ecological divergence (McKinnon *et al.*, 2004; Boughman *et al.*, 2005) and secondarily on differences in colouration (Boughman, 2001; McKinnon *et al.*, 2004; Boughman *et al.*, 2005). Similarly, in some cichlid species, reproductive isolation appears to depend on the operation of visual cues (Seehausen *et al.*, 1997) where deterioration of environmental cues (e.g. lake eutrophication and the reduced transmission of light) is associated with increased hybridization.

Our study found no evidence for reinforcement (i.e. increased behavioural isolation in areas of sympatry). The lack of reinforcement may be due to the fact that sympatric populations are not stable over long enough periods of time for selection to produce increased levels of behavioural isolation. In this study, the zone of sympatry fluctuated where some populations were sympatric at one census period, but allopatric at another census period. Continued long-term censuses of these sites will indicate the degree to which these populations are consistently sympatric.

This study also provides little evidence for genic isolation in the F1 hybrid stage. Instead we found high hatching success and high survival to adulthood when raised in stock tanks at 2 ppt. The idea that genic isolation is not important in the early stages of speciation in teleosts is supported by two comparative studies (darters – Mendelson, 2003; sunfish – Bolnick & Near, 2005) as well as studies on individual species/population pairs (sticklebacks – Hatfield & Schluter, 1999; guppy – Alexander & Breden, 2004). However, genic isolation has been demonstrated numerous times in other groups (plants, Fishman & Willis, 2001; Brandvain & Haig, 2005; Lepidoptera, Presgraves, 2002 and *Drosophila*, see Coyne & Orr, 2004 for a review), and there are a few examples in teleosts as well. In whitefish (*Coregonus*), F1 and backcrossed animals have been shown to experience significantly higher mortality than either parental strain (Lu & Bernatchez, 1998; Rogers & Bernatchez, 2006). Similarly, a study on hybridization among guppy populations

examined both the F1 and F2 stages and found evidence for reduced fitness in the F2 stage (Russell & Magurran, 2006). It has also been suggested that hybridization between marine and landlocked sticklebacks (*G. aculeatus*) produces sterile F1 males (Honma & Tamura, 1984).

The apparent lack of evidence for genic isolation in some fish groups may be due to the fact that: (1) only a small number of taxa have been studied; (2) some studies only examine the survival of embryos and ignore later reproductive stages; (3) survival in the F2 and back-crossed animals is often not measured due to the fact that many fish require at least a full year to attain reproductive maturity; and (4) survival under natural conditions is infrequently studied. These reservations apply equally to this work. We primarily investigated survival in F1 animals at early life-history stages. Although we were able to raise hybrids to adulthood at one salinity level, we were unable to examine their survival over the range of relevant salinities. Furthermore, we have not yet raised them under natural conditions where they would be exposed to predators and other stressful factors. Currently, we are investigating genic isolation in F2 and back-crossed animals.

Similarly, we found little evidence that F1 hybrids were differentially affected by salinity. At early life-history stages, we found high hatching success and larval survival of F1 animals at all salinities – even at the higher salinities where *L. goodei* had low hatching success. Unfortunately, we were unable to raise F1 animals to adulthood in stock tanks at all salinities where we found the greatest evidence for effects of salinity. Thus we were unable to test whether hybrid fitness varied across a range of salinities. This is unfortunate because ecologically dependent post-zygotic isolation is a unique prediction of ecological speciation (Rundle & Nosil, 2005). Environmentally dependent hybrid inferiority has been demonstrated in some systems (Jiggins *et al*., 2001). In *G. aculeatus*, F1 backcrosses to benthic parents have higher fitness in benthic habitats than F1 backcrosses to limnetic parents (Rundle, 2002) indicating that reduced hybrid fitness is dependent on ecological conditions in this group.

In conclusion, our study found strong evidence that *L. goodei* and *L. parva* are differentially adapted to salinity where *L. goodei* is very tolerant of fresh water and *L. parva* is tolerant of brackish water. However, we found this effect only when animals were raised to adulthood in stock tanks and forced to overwinter or when *L. goodei* eggs and fry were raised at salinities above 10 ppt. The interaction between salinity and temperature may be the critical factor that differentiates habitat space between *L. goodei* and *L. parva*. We also found strong evidence for prezygotic, behavioural isolation between *L. goodei* and *L. parva*. We found no evidence for genic isolation (i.e. hybrid inviability) in F1 animals. Hatching success, probability of surviving through the eating stage, and survival to adulthood were intermediate between the two parent species. However, we are currently investigating survival of F2 and back-crossed animals.

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