AN EXPERIMENTAL TEST OF CHARACTER DISPLACEMENT'S ROLE IN PROMOTING POSTMATING ISOLATION BETWEEN CONSPECIFIC POPULATIONS IN CONTRASTING COMPETITIVE ENVIRONMENTS

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Ecological character displacement takes place when two closely related species co-occur in only part of their geographical range, and selection to minimize competition between them promotes divergence in resource-use traits in sympatry but not in allopatry. Because populations sympatric with the heterospecific competitor will experience a different competitive environment than conspecific populations in allopatry, conspecific populations from these two competitive environments will also diverge in resource traits as an indirect consequence of interspecific ecological character displacement. Ultimately, ecologically dependent postmating isolation may arise between conspecific populations from these divergent competitive environments if offspring produced by matings between them are competitively inferior in either type of competitive environment. Yet, there are no direct tests of character displacement's role in initiating such postmating isolation. Here, we present a test by comparing the phenotypes and performances of spadefoot toad tadpoles produced from between-competitive-environment (BCE) matings versus those produced from within-competitive-environment (WCE) matings. When raised with naturally occurring competitors, BCE offspring grew significantly less and were significantly smaller than WCE offspring. BCE offspring generally performed worse even when raised alone, suggesting that they may have harbored intrinsic genetic incompatibilities. Moreover, the difference in growth and body size of BCE versus WCE offspring was significantly greater when each was raised with competitors than when each was raised alone, suggesting that BCE tadpoles were competitively inferior to WCE tadpoles. Presumably, this enhanced difference arose because BCE tadpoles produced an intermediate resource-use phenotype that is less well adapted to either competitive environment. Because larval size is under strong, positive, directional selection, reduced growth and size of BCE offspring may diminish gene flow between populations in divergent competitive environments, thereby generating postmating isolation. Thus, postmating isolation between conspecific populations, and possibly even speciation, may arise as a by-product of interactions between species.

KEY WORDS: Competition, ecological character displacement, ecological speciation, ecologically dependent postmating isolation, spadefoot toads.

When two closely related species co-occur in only part of their geographical range, they often differ in resource use and associated traits in sympatry but not in allopatry (Fig. 1A; see Brown and Wilson 1956; Schluter 2000; Dayan and Simberloff 2005). Such "ecological character displacement" is thought to arise when competition between similar heterospecific individuals imposes divergent directional selection on each species' resource use (reviewed in Schluter 2000; Day and Young 2004).
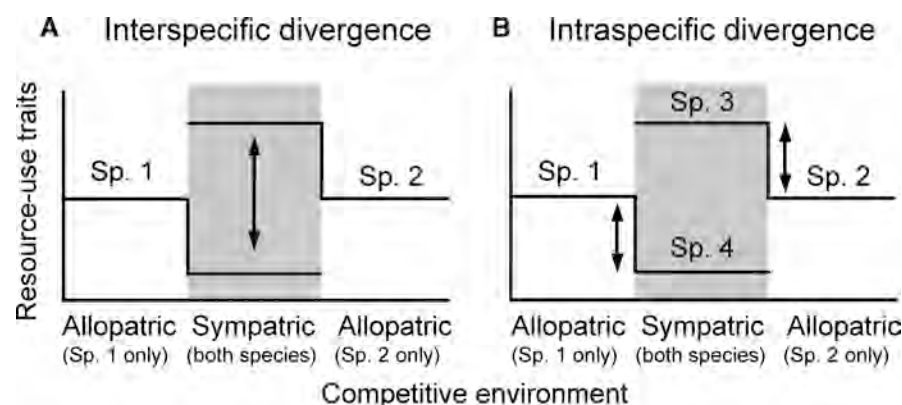
Figure 1. Character displacement promotes divergence between interacting species and, possibly, the formation of new species. (A) When two closely related species (Sp. 1 and Sp. 2) co-occur in only part of their geographical range (indicated by the shading), selection to minimize competition may promote divergence between them in resource-use traits in sympatry with the heterospecific competitor but not in allopatry (indicated by the doubled-headed arrow). (B) An indirect consequence of character displacement is that individuals in sympatry with the heterospecific competitor will experience a different competitive environment than conspecifics in allopatry. As a result, conspecific populations from these two environments may diverge in resource-use traits (indicated by the two, smaller, doubled-headed arrows). Consequently, postmating isolation may arise between conspecific populations in divergent environments if offspring produced by matings between them are disfavored because they are competitively inferior in either type of competitive environment. This process may eventually promote the formation of two new species (Sp. 3 and Sp. 4).

An indirect consequence of character displacement is that individuals in sympatry with the heterospecific competitor will experience a different competitive environment than conspecifics in allopatry. Consequently, conspecific populations in these two types of environments may diverge in resource-use traits (Fig. 1B). Between-competitive-environment (BCE) offspring (i.e., offspring produced by matings between individuals from different competitive environments) may therefore produce an intermediate phenotype that is less well adapted to either competitive environment than that produced by within-competitive-environment (WCE) offspring (i.e., offspring produced by matings between individuals from the same competitive environment). If such maladaptation limits gene flow between populations in different competitive environments, it may constitute "ecologically dependent postmating isolation," which occurs when "hybrids" (whether hybridization is between populations or species) are at a selective disadvantage because they are intermediate in phenotype and an intermediate niche is lacking (Rice 1987; Hatfield and Schluter 1999; Rundle 2002). Moreover, because of this reduced gene flow, populations in divergent selective environments may begin to evolve independently of each other and thereby accumulate alleles that are incompatible with genomes from the alternative environment (reviewed in Coyne and Orr 2004). Ultimately, these barriers to gene flow between populations in different competitive environments may promote "ecological speciation" (Schluter 2001).

Despite longstanding arguments that ecological character displacement may initiate speciation between conspecific populations in contrasting competitive environments, this possibility remains virtually unexplored empirically (Rundle and Nosil 2005).

One way to test this theory is to evaluate whether, in a species undergoing character displacement, BCE offspring have reduced fitness relative to WCE offspring, and whether any such maladaptation arises because the former are at a competitive disadvantage in either type of competitive environment. Here, we present such a test in spadefoot toad tadpoles.

STUDY SYSTEM

Mexican (*Spea multiplicata*) and Plains spadefoot toads (*S. bombifrons*) co-occur below 1350 m elevation in southeastern Arizona and southwestern New Mexico; only *S. multiplicata* occur at higher elevations (Pfennig et al. 2006). Both species potentially develop into either a small tadpole with normal sized jaw muscles specialized for feeding on detritus on the pond bottom (the "omnivore" morph), or a larger tadpole with greatly enlarged jaw muscles specialized for feeding on anostracan fairy shrimp in open water (the "carnivore" morph [Bragg 1965; Pomeroy 1981]). Shrimp ingestion triggers development of carnivores from omnivores (Pomeroy 1981; Pfennig 1990).

These two species undergo ecological character displacement in larval trophic morphology (Pfennig and Murphy 2000, 2002, 2003; Pfennig et al. 2006, 2007). When each species occurs alone, they produce similar frequencies of both morphs (Pfennig and Murphy 2003; Pfennig et al. 2006). By contrast, when they occur together, *S. bombifrons* produce mostly carnivores, whereas *S. multiplicata* produce mostly omnivores (Pfennig and Murphy 2003; Pfennig et al. 2006). Moreover, *S. multiplicata* undergo an evolutionary shift in developmental mechanism: in allopatry, *S. multiplicata* maintain plasticity to produce both morphs, but

in adjacent sympatry, they become canalized to produce mostly omnivores (Pfennig and Murphy 2000, 2002).

Divergence in morph production mirrors competitive differences between species. Sympatric *S. multiplicata* are superior competitors for detritus, whereas sympatric *S. bombifrons* are superior competitors for shrimp (Pfennig and Murphy 2000). These competitive differences reflect species-specific differences in behavior. Because they spend more time in the water column, *S. bombifrons* forage more effectively for shrimp (Pfennig and Murphy 2000). In contrast, *S. multiplicata* spend more time grazing on the pond bottom and, thus, forage more effectively for detritus (Pfennig and Murphy 2000).

These shifts between species in morph production have likely evolved because of resource competition imposed by the other species. Individual *S. multiplicata* that are the most similar to *S. bombifrons* in resource use perform the worst when competing with *S. bombifrons* for food, indicating that selection favors divergence between species (Pfennig et al. 2007). Moreover, in the presence of the other species, allopatric individuals of each species facultatively express an alternative phenotype less like the other species' phenotype (Pfennig and Murphy 2000, 2002). Because these experimentally demonstrated niche shifts mirror the canalized niche shifts observed in the field, they provide experimental support for character displacement.

SPECIFIC PREDICTIONS

In this study, we used *S. multiplicata* as our focal species. Based on the earlier work described above, we predicted that BCE offspring (i.e., offspring produced from a sympatric parent mated to an allopatric parent) would perform significantly worse than WCE offspring (i.e., offspring produced from a sympatric parent mated to a sympatric parent or an allopatric parent mated to an allopatric parent). In an allopatric competitive environment (i.e., in ponds where only *S. multiplicata* are present), BCE tadpoles should not grow as well as WCE tadpoles derived from allopatry. *Spea multiplicata* derived from allopatry are more likely than those from sympatry to produce carnivores (Pfennig and Murphy 2000, 2002), and, perhaps, also to produce a version of the carnivore morph that preys more effectively on shrimp (Pomeroy 1981). Because shrimp are the more nutritious resource (Pfennig 2000), those individuals that can best use shrimp should exhibit higher growth, which correlates with higher fitness in this system (reviewed in Pfennig et al. 2007). Thus, BCE offspring should perform significantly worse than WCE offspring in an allopatric environment, because BCE offspring should be poorer at preying on shrimp, the more nutritious resource in this system.

Similarly, in a sympatric competitive environment (i.e., in ponds where both species are present), BCE tadpoles should not grow as well as WCE tadpoles derived from sympatry. Neither cross type should have high access to shrimp in a sym-

patric environment, because *S. bombifrons* is the superior competitor for shrimp (Pfennig and Murphy 2000). At the same time, *S. multiplicata* derived from a sympatric competitive environment are canalized to produce mostly omnivores (Pfennig and Murphy 2000, 2002), which are more effective foragers on detritus (Pomeroy 1981). Thus, BCE offspring should perform significantly worse than WCE offspring in a sympatric environment, because BCE offspring should be competitively inferior on either resource type.

Materials and Methods

We performed two sets of experiments to test the above predictions and thereby evaluate character displacement's potential role in promoting postmating isolation. In the first experiment, we investigated whether BCE offspring produce an intermediate resource-use phenotype. Such a result might suggest that any maladaptation observed in the BCE offspring specifically results from ecologically based divergent selection (e.g., see Hatfield and Schluter 1999; Rundle 2002). In the second experiment, we asked whether fitness of BCE offspring is lower than that of WCE offspring, and whether any such reduction results from reduced competitive ability of BCE offspring. Our fitness proxies—growth to, and body size at, metamorphosis—correlate positively with several fitness components in this system; larger, faster growing individuals are favored by both natural and sexual selection (reviewed in Pfennig and Pfennig 2005; Pfennig et al. 2007). We contrasted size of BCE versus WCE offspring after each had been reared (1) in isolation, to determine if any reduction in size of BCE offspring (relative to WCE offspring) might have resulted from intrinsic genetic incompatibilities, and (2) in the two different parental competitive environments, to determine if any reduction in size of BCE offspring might have resulted from reduced competitive ability, which may ultimately cause ecologically dependent postmating isolation.

COMPARING PHENOTYPES OF BCE VERSUS WCE OFFSPRING

Ecologically dependent postmating isolation occurs when BCE offspring are at a competitive disadvantage in either parental environment, because they are intermediate in phenotype and an intermediate resource is lacking (Rice 1987; Hatfield and Schluter 1999; Rundle 2002). We therefore examined whether BCE offspring produce a resource-use phenotype that is intermediate between that produced by WCE offspring from each of the two parental competitive environments. Specifically, we compared three morphological and behavioral characters among WCE tadpoles, BCE tadpoles, and *S. bombifrons* tadpoles.

We began by collecting adult *S. multiplicata* at high elevation (allopatric) and low elevation (sympatric) sites in the San Simon Valley of Cochise County, Arizona, and adjacent Hidalgo
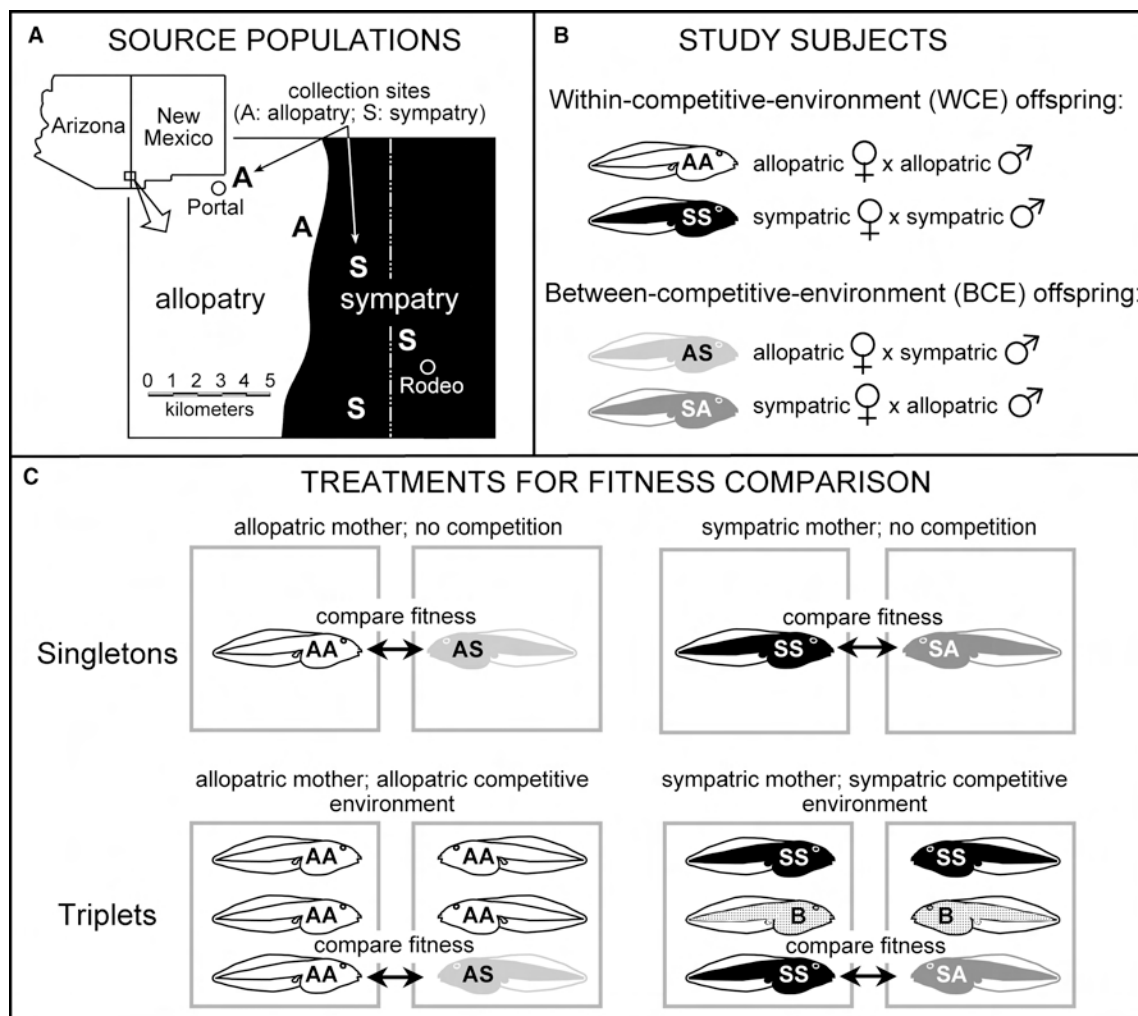
Figure 2. Diagram of methods. (A) Adult *S. multiplicata* were collected from five different populations, three of which were locally "sympatric" with *S. bombifrons* in a region (in black) where the distributions of both species overlap, and two of which were from a region (in white) outside the distribution of *S. bombifrons* and thus did not co-occur with it. These populations were considered "allopatric." (B) These animals were bred to create tadpoles of four different cross types, based on the allopatric/sympatric status of their parents' source populations. (C) For the fitness comparison experiment, tadpoles were either reared alone (as "singletons") or with two competitors (as "triplets"; B: *S. bombifrons*). For both triplets and singletons, we compared growth and overall body size (fitness proxies) of between-competitive-environment (BCE) offspring versus within-competitive-environment (WCE) offspring whose mothers were from the same competitive environment (i.e., allopatry or sympatry).

County, New Mexico (Fig. 2A). Allopatric sites ($N = 2$) were 3 km apart, sympatric sites ($N = 3$) were 3–7 km apart, and allopatric and sympatric sites were 3–11 km apart (Fig. 2A). Character displacement has been documented between *S. multiplicata* and *S. bombifrons* from these sympatric populations (Pfennig and Murphy 2000, 2002, 2003; Pfennig et al. 2006, 2007).

We created 15 full sibships of *S. multiplicata* by pairing adults from allopatry and sympatry according to four different cross types (Fig. 2B): (1) allopatric female × allopatric male ("AA" cross type; three sibships; for each sibship, the mother and father were from different allopatric populations); (2) sympatric female × sympatric male ("SS" cross type; four sibships; for each sibship, the mother and father were from different sympatric pop-

ulations); (3) allopatric female × sympatric male ("AS" cross type; four sibships); (4) sympatric female × allopatric male ("SA" cross type; four sibships). AA and SS cross types generated WCE offspring, whereas AS and SA cross types generated BCE offspring (Fig. 2B). We also created four full sibships of *S. bombifrons* by breeding four pairs of *S. bombifrons* from the same populations as the sympatric *S. multiplicata*. For both species, sibling tadpoles were kept in separate nursery tanks and fed fish food ad libitum until needed.

First, we contrasted trophic morphology of WCE tadpoles, BCE tadpoles, and *S. bombifrons* tadpoles. To do so, we randomly selected similarly sized, three-day-old tadpoles from all 19 sibships and placed two siblings together (to induce competition) in

replicate tanks (28 × 18 × 10 cm) filled with 6 L of dechlorinated water. We fed these tadpoles live brine shrimp and detritus (dead shrimp) ad libitum. After 12 days, the tadpoles were killed, and we measured three diagnostic, morphological characters using the procedures in Pfennig et al. (2007): (1) width of the orbitohyoideus (OH) muscle, (2) width of the interhyoideus (IH) muscle, and (3) shape of the keratinized mouthparts (MP). We combined the three characters (MP, residuals of log OH and log IH regressed on log snout-vent length [SVL]) for each tadpole into a single multivariate shape variable (the "morphological index") by performing a principal component (PC) analysis (we used PC1, which explained > 60% of the variance in the three trophic characters). For the analysis, we used the mean morphological index for each pair of tankmates. We used a one-way ANOVA to compare these means from 44 pairs of AA tadpoles, 54 pairs of AS tadpoles, 47 pairs of SS tadpoles, 43 pairs of SA tadpoles, and 44 pairs of *S. bombifrons* tadpoles. We specifically predicted (see "Specific predictions") that AS tadpoles would have a smaller mean morphological index (less carnivore-like) than AA tadpoles; however, we predicated that SA tadpoles would have a larger mean morphological index (more carnivore-like) than SS tadpoles, but a smaller mean morphological index (less carnivore-like) than *S. bombifrons*.

Next, using the procedures in Pfennig et al. (2007), we measured the propensity to eat shrimp for each group. We randomly selected similarly sized, 12-day-old tadpoles from all 19 sibships and placed each tadpole alone in a small, round, individually numbered, opaque plastic container (12 cm diameter × 6 cm deep) containing 600 ml of dechlorinated tap water. Each tadpole acclimated to its new surroundings within 24 h, during which time it was given crushed fish food ad libitum. We then placed into each container three live brine shrimp (*Artemia* sp., 10 mm total length). Brine shrimp are similar to the fairy shrimp on which *Spea* prey in natural ponds (Pomeroy 1981). An observer then recorded how long each tadpole took to consume all three shrimp in its container (none of the tadpoles used in this experiment had previously encountered shrimp). Using a one-way ANOVA on transformed (square root) data from 84 AA tadpoles, 84 AS tadpoles, 166 SS tadpoles, 84 SA tadpoles, and 140 *S. bombifrons* tadpoles (all of which were similarly satiated at the beginning of the experiment), we determined if the five groups differed in time to eat shrimp. We specifically predicted (see "Specific predictions") that AS tadpoles would eat shrimp more slowly (less carnivore-like) than AA tadpoles; however, we predicted that SA tadpoles would eat shrimp more quickly (more carnivore-like) than SS tadpoles, but more slowly (less carnivore-like) than *S. bombifrons*.

Finally, we modified the methods in Pfennig and Murphy (2000) to measure how similar *S. multiplicata* tadpoles in each group were in tendency to graze. We randomly selected from the nursery tanks 154 15-day-old tadpoles from all 15 *S. multiplicata*

sibships and placed each tadpole individually in a clear plastic aquarium (34 × 22 × 10 cm) containing 6 L of dechlorinated water. After a 10-min acclimation period, we observed each tadpole continuously for 10 min and categorized its behavior as grazing (feeding on the bottom or surface of the water), swimming (moving through the water column but not obviously feeding), or resting (motionless). Using a one-way ANOVA on transformed (arcsine-square root) proportion data from 37 AA tadpoles, 40 AS tadpoles, 38 SS tadpoles, and 39 SA tadpoles, we determined if the four groups differed in the proportion of time spent grazing. We specifically predicted (see "Specific predictions") that AS tadpoles would graze more (less carnivore-like) than AA tadpoles; however, we predicted that SA tadpoles would graze less (more carnivore-like) than SS tadpoles.

EVALUATING THE FITNESS OF BCE VERSUS WCE OFFSPRING

Next, we asked whether fitness of BCE offspring is lower than that of WCE offspring, and whether any such reduction in performance results from reduced competitive ability of BCE offspring. To create focal tadpoles, we bred the adults from the previous experiment (see "Comparing phenotypes of BCE versus WCE offspring") for a second time to create 15 new sibships of *S. multiplicata* and four new sibships of *S. bombifrons* (19 sibships total). We created the same four *S. multiplicata* cross types as before (Fig. 2B).

Sixteen days after the tadpoles hatched, we randomly selected as focal individuals 23 tadpoles from each of the above 15 *S. multiplicata* sibships (345 tadpoles total). We then used a 26-gauge hypodermic needle to inject a fluorescent orange elastomer (Northwest Marine Technology, Inc., Shaw Island, WA) into the dorsal tail membrane of each tadpole. Once injected, the material formed an inert strip (approximately 1 mm wide by 5 mm long) visible under ultraviolet light. The marking procedure did not affect larval mortality. We then obtained an estimate of the initial mass of each marked tadpole by blotting the live tadpole on tissue and weighing it on an electronic balance to the nearest 0.001 g.

We randomly assigned focal tadpoles from each cross type to one of two treatment groups (Fig. 2C): (1) singletons ($N = 182$ tanks), in which a focal tadpole was reared alone, and (2) triplets ($N = 160$ tanks), in which a focal tadpole was reared with two competitors. Triplets mimicked the two competitive environments (allopatry and sympatry), with the source population and species identity of the competitor tadpoles reflecting the source population of the focal tadpole's mother. For those focal tadpoles whose mother was from an allopatric population (i.e., AA and AS crosses; $N = 80$ tanks), the competitors were both AA tadpoles (nonsiblings to each other and to the AA focal tadpole). This situation mimicked a natural allopatric environment in which a tadpole would encounter only AA tadpoles. By contrast, for those focal

tadpoles whose mother was from a sympatric population (i.e., SS and SA crosses; $N = 80$ tanks), one competitor was an SS tadpole (a nonsibling to the SS focal tadpole), and the other was an *S. bombifrons*. This situation mimicked a natural sympatric environment in which a tadpole would encounter both SS tadpoles and *S. bombifrons*, often in equal numbers (the design of this experiment is analogous to the same host vs. different host comparative approach for evaluating ecological speciation introduced by Funk [1998]).

For both triplets and singletons, we compared body size and growth as surrogates of fitness in the BCE versus WCE offspring. These comparisons involved offspring with mothers from the same competitive environment (Fig. 2C). That is, we contrasted AA versus AS tadpoles (both mothers from the allopatric environment) and SS versus SA tadpoles (both mothers from the sympatric environment). These comparisons allowed us to contrast the expected fitness of two hypothetical females from the same competitive environment, one of which mates with a male from her competitive environment and the other of which mates with a migrant male from the alternative environment. (Females choose mates in this system [K. Pfennig 2000]) and probably disperse less than males [K. Pfennig; pers. comm.]). We sought to ascertain whether the latter female would pay a fitness penalty because of ecological selection against her offspring. Specifically, we used a paired design to compare performance of size-matched BCE and WCE focal tadpoles that were placed in adjacent tanks. These tadpoles experienced similar microenvironments, and their performance was contrasted at the end of the experiment (see Fig. 2C).

We did not compare performance of tadpoles with mothers from different competitive environments (i.e., AA vs. SA tadpoles or SS vs. AS tadpoles) because the markedly different competitive environments they experienced in our triplet treatment would yield uncontrolled comparisons. Indeed, *S. multiplicata* reared in the sympatric environment were smaller than those in the allopatric environment (see "Results"), presumably because of the presence of *S. bombifrons*, which are more effective predators on shrimp (see "Specific predictions").

Both triplets and singletons were placed in tanks ($28 \times 18 \times 10$ cm) with 6 L of dechlorinated tap water and arrayed on wire racks. All tanks were kept in the same room at $26°$C on a 14:10 light:dark photoperiod. The per capita amount of food provided to both triplets and singletons was identical. Specifically, triplets each received 30 ml of water saturated with brine shrimp nauplii (*Artemia*) once a day (*Artemia* resemble the fairy shrimp on which *Spea* feed in natural ponds) and 30 mg of crushed fish food every other day (fish food simulates in form and nutrition the detritus on which *Spea* feed in natural ponds; see Pfennig et al. 2006). Each singleton tank received one-third of this amount of food. Shrimp and detritus had disappeared by the time the tadpoles

were fed again, ensuring that competition had taken place in the triplets.

Because tadpoles stop feeding and therefore competing as they approach metamorphosis, we ended the triplet experiment when the first metamorphs appeared (on day 16; all tadpoles were at or near metamorphosis at this time). We obtained an estimate of the final mass of all marked tadpoles (triplets and singletons) by using the same procedures as for estimating initial mass. We then calculated each focal tadpole's increase in mass over the 16-d duration of the experiment by subtracting its initial wet mass from its final wet mass.

On day 16, we also recorded SVL of each focal tadpole in the triplet treatment (to the nearest 0.01 mm) using handheld digital calipers. Singletons were each allowed to proceed to metamorphosis (operationally defined as the emergence from the body of at least one front limb) before measuring their SVL (i.e., mass was recorded on the same date for triplets and singletons, but SVL was recorded on different dates). Anurans lose size during metamorphosis. Thus, because SVL was measured just before metamorphosis for triplets, rather than being measured at metamorphosis as for singletons, SVL of triplets tended to be larger than that of singletons (see "Results").

We sought to determine if BCE offspring were smaller in final body size and increased significantly less in mass than their matched WCE offspring. We also sought to determine if BCE offspring specifically suffered from reduced competitive ability; that is, if the difference in body size and growth of BCE versus WCE offspring was greater when each was raised with competitors compared to when each was raised alone. To test these predictions, we used separate matched-pairs *t*-tests for triplets and singletons to compare body size (SVL) and change in mass of each matched pair (see Fig. 2C). To test for the possible presence of ecologically dependent postmating isolation, we also used a one-tailed *t*-test to determine if, within each competitive environment (sympatry or allopatry), the difference (WCE − BCE) in body size and growth was greater when each type of tadpole was raised with competitors (i.e., in triplets) versus when raised alone (i.e., in singletons). We used a one-tailed test for this comparison because of our a priori observation that the BCE offspring produced an intermediate resource-use phenotype (see Results), which we had predicted would make them competitively inferior to WCE offspring (see "Specific predictions").

Finally, because triplets were larger than singletons by the end of the experiment, the likelihood of observing larger performance differences was greater for triplets than for singletons. Thus, to determine if the difference in performance (WCE offspring − BCE offspring) was indeed greater for triplets than for singletons (and thereby to establish whether BCE offspring were competitively inferior to WCE offspring), we compared growth rate of each cross type in the two treatment groups (growth rate is not affected

by differences between groups in absolute size). We estimated growth rate for each focal tadpole as

$$(\log(\text{final wet mass}) - \log(\text{initial wet mass}))/16\,\text{days}$$

where 16 days was the duration of the experiment. As before, to test for the possible presence of ecologically dependent post-mating isolation, we also used a one-tailed t-test to determine if, within each competitive environment (sympatry or allopatry), the difference (WCE − BCE) in growth rate was greater in triplets than in singletons.

Results

COMPARING PHENOTYPES OF BCE VERSUS WCE OFFSPRING

Phenotypic comparisons are shown in Figure 3. In morphological index (Fig. 3A), *S. multiplicata* were less carnivore-like than were *S. bombifrons*, and AS tadpoles were significantly less carnivore-like than were AA tadpoles, matching our predictions. Contrary to our expectation, however, SS and SA tadpoles did not differ. In time to eat shrimp (Fig. 3B), *S. multiplicata* were less carnivore-like than were *S. bombifrons*, and *S. multiplicata* tadpoles from sympatric mothers (i.e., SS and SA tadpoles) and were less carnivore-like than were *S. multiplicata* tadpoles from allopatric mothers (i.e., AA and AS tadpoles), again, matching our predictions. Within each competitive environment, however, WCE and BCE offspring did not differ significantly in shrimp eating times. Finally, in time spent grazing (Fig. 3C), the four cross types did not differ.

EVALUATING THE FITNESS OF BCE VERSUS WCE OFFSPRING

As summarized in Figure 4, BCE offspring generally grew significantly less than WCE offspring and were smaller, regardless of whether they were raised alone or with competitors, and regardless of which competitive environment their mother was derived from (sympatry or allopatry).

SS singletons were significantly larger in mean ($\pm$ SEM) overall body size (SVL; 15.30 ± 0.14 mm) than SA singletons (14.96 ± 0.14 mm, difference in SVL, SS − SA $= 0.34 \pm 0.17$ mm, $t_{44} = 2.02$, $P = 0.0497$; Fig. 4B). By contrast, AA singletons (15.13 ± 0.18 mm) did not differ from AS singletons (14.87 ± 0.17 mm, AA − AS $= 0.26 \pm 0.26$ mm, $t_{37} = 1.09$, $P = 0.2817$; Fig. 4A). When we combined offspring from sympatric and allopatric mothers, however, SVL of all WCE tadpoles (15.29 ± 0.11 mm) was significantly greater than that of all BCE tadpoles (14.93 ± 0.11 mm, WCE − BCE $= 0.35 \pm 0.15$ mm, $t_{174} = 2.28$, $P = 0.0236$). AA triplets (16.91 ± 0.27 mm) were significantly larger in SVL than AS triplets (16.15 ± 0.22 mm, AA − AS $= 0.76 \pm 0.23$ mm, $t_{37} = 3.34$, $P = 0.0019$; Fig. 4A),
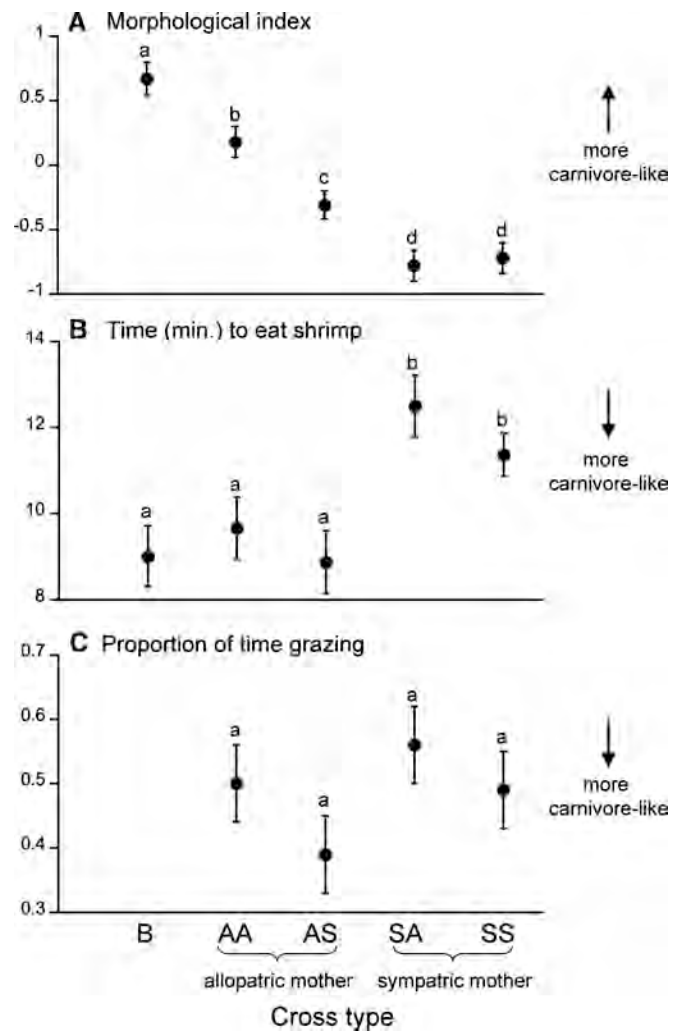

Figure 3. Comparison of (A) morphological and (B, C) behavioral characters for *S. bombifrons* ("B"), within-competitive-environment (WCE) offspring of *S. multiplicata* ("AA" and "SS" cross types; see Fig. 2B), and between-competitive-environment (BCE) offspring of *S. multiplicata* ("AS" and "SA" cross types). Within each graph, cross types with different letters are significantly different from one another ($P < 0.05$; Tukey–Kramer HSD). See text for Specific predictions.

and SS triplets (15.82 ± 0.23 mm) were significantly larger in SVL than SA triplets (15.10 ± 0.28 mm, SS − SA $= 0.73 \pm 0.31$ mm, $t_{36} = 2.35$ $P = 0.0244$; Fig. 4B). Moreover, when we combined all tadpoles, the difference in SVL (WCE offspring − BCE offspring) was significantly greater in triplets (0.74 ± 0.17) than in singletons (0.30 ± 0.16, triplets − singletons $= 0.44 \pm 0.23$ mm, $t_{156} = 1.89$, one-tailed $P = 0.0301$).

AA singletons (0.822 ± 0.018 g) increased significantly more in mass than did AS singletons (0.770 ± 0.022 g, difference in increase in mass, AA − AS $= 0.052 \pm 0.023$ g, $t_{39} = 2.291$, $P = 0.0275$; Fig. 4C), and SS singletons (0.879 ± 0.023 g) increased significantly more in mass than did SA singletons (0.775 ± 0.029
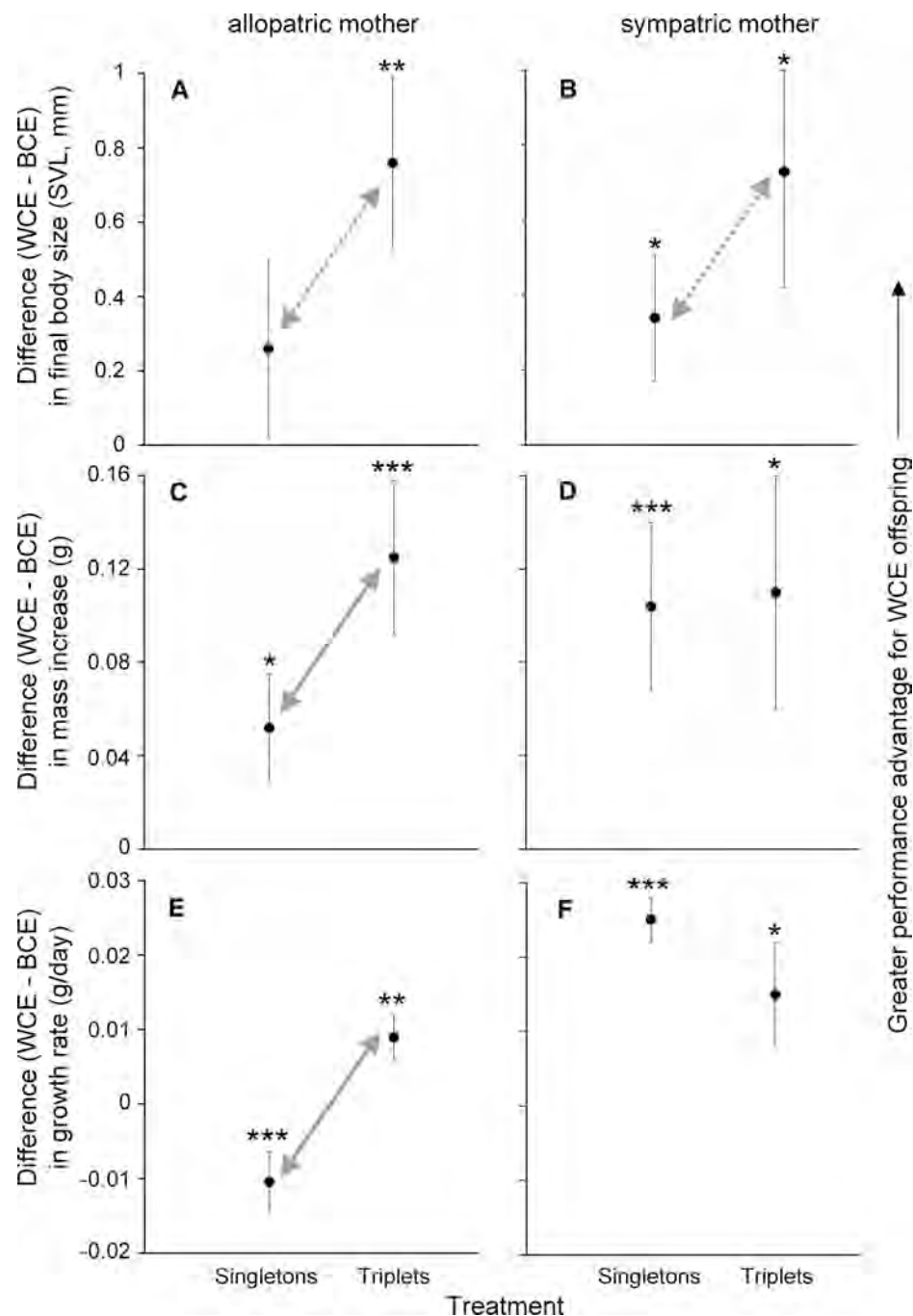
Figure 4. Relative performance of within-competitive-environment (WCE) offspring versus between-competitive-environment (BCE) offspring, as revealed by three fitness proxies: (A, B) final body size (i.e., snout-vent length, SVL), (C, D) growth (i.e., overall increase in wet mass), and (E, F) growth rate (i.e., rate of increase in wet mass). Each focal individual was produced by either an allopatric mother (A, C, E) or a sympatric mother (B, D, F) and was raised either alone (as singletons) or with competitors found in the maternal environment (as triplets). For each response measure, more positive values indicate a larger performance advantage for WCE offspring. Asterisks denote mean performance differences that are significantly different from zero, the value expected if BCE and WCE offspring were equivalent in performance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Double-headed arrows denote that the mean performance difference (WCE offspring − BCE offspring) was significantly greater in triplets than in singletons (dashed double-headed arrows indicate that the performance difference was significantly greater in triplets than in singletons when allopatric and sympatric tadpoles were combined), suggesting that BCE offspring specifically suffered from reduced competitive ability.

g, SS − SA = 0.104 ± 0.036 g, t_{44} = 2.885, P = 0.0063; Fig. 4D). Similarly, AA triplets (0.939 ± 0.033 g) increased significantly more in mass than did AS triplets (0.814 ± 0.026 g, AA − AS = 0.125 ± 0.033 g, t_{37} = 3.785, P = 0.0006; Fig. 4C), and SS triplets (0.742 ± 0.030 g) increased significantly more in mass than did SA triplets (0.692 ± 0.036 g, SS − SA = 0.112 ± 0.050 g, t_{36} = 2.248, P = 0.0310; Fig. 4D). Moreover, for allopatric mothers, the difference in increase in mass (AA tadpoles − AS tadpoles) was significantly greater in triplets than in singletons (triplets − singletons = 0.073 ± 0.039 g, t_{71} = 1.882, one-tailed P = 0.0319).

Finally, AA singletons grew significantly more slowly (0.124 ± 0.003 g/d) than did AS singletons (0.136 ± 0.003 g/d, difference in growth rate, AA − AS = −0.012 ± 0.017 g/d, t_{39} = 4.305, P < 0.0001; Fig. 4E). By contrast, SS singletons grew significantly faster (0.140 ± 0.004 g/d) than did SA singletons (0.115 ± 0.004 g/d, SS − SA = 0.025 ± 0.003 g/d, t_{44} = 9.666, P < 0.0001; Fig. 4F). AA triplets grew significantly faster (0.142 ± 0.005 g/d) than did AS triplets (0.133 ± 0.005 g/d, AA − AS = 0.009 ± 0.003 g/d, t_{37} = 2.907, P = 0.0066; Fig. 4E), and SS triplets grew significantly faster (0.124 ± 0.004 g/d) than did SA triplets (0.110 ± 0.005 g/d, SS − SA = 0.014 ± 0.007 g/d, t_{36} = 2.199, P = 0.0346; Fig. 4F). Moreover, for allopatric mothers, the difference in growth rate (AA tadpoles − AS tadpoles) was significantly greater in triplets than in singletons (triplets – singletons = 0.019 ± 0.005 g, t_{71} = 3.887, one-tailed P < 0.0001).

Discussion

Trait evolution stemming from competition between species for resources—ecological character displacement—has long been viewed as an important cause of phenotypic differences between interacting species (Schluter 2000). An indirect consequence of character displacement, however, is that offspring produced by matings between conspecifics that have evolved in sympatry with the heterospecific competitor and those that have evolved in allopatry with no competitor present may be poorly suited for either parental competitive environment. If such BCE offspring are sufficiently disfavored (relative to WCE offspring), conspecifics in different competitive environments may be partially reproductively isolated from each other through hybrid inviability. Ultimately, character displacement may trigger ecological speciation between conspecific populations in contrasting competitive environments (Fig. 1B). Yet, to date, there have been no direct tests of this theory (Rundle and Nosil 2005).

Our study contributes to filling this void. We evaluated ecological character displacement's role in promoting postmating isolation of sympatric and allopatric populations by contrasting the performance of offspring produced from matings of spadefoot toads representing populations from the same versus divergent competitive environments (Fig. 2A). These environments are

those that either have or have not undergone ecological character displacement with a heterospecific. We found that BCE tadpoles grew significantly less and were significantly smaller than WCE tadpoles (Fig. 4). The smaller body size of BCE offspring would likely have important fitness consequences, given that body size is under strong, positive, directional selection (Pfennig et al. 2007). Indeed, the observed average 6% reduction in body size (SVL) among BCE tadpoles (relative to WCE tadpoles) may even cause postmating isolation between populations in different competitive environments. Based on sized-based selection estimates obtained from earlier studies of these populations (Pfennig and Pfennig 2005), if this same difference in SVL were translated directly into a similar difference in adult body size, BCE females would suffer a 16% reduction in fecundity (from 1250 eggs to 1050 eggs), which may suffice to reduce gene flow substantially between allopatric and sympatric populations.

BCE offspring had lower growth rates and were smaller than WCE offspring with one exception: AS singletons had a higher growth rate than AA singletons (Fig. 4E). These BCE offspring of allopatric females likely grew faster than WCE offspring of allopatric females because AS tadpoles had a father derived from the sympatric environment (Fig. 2B). Tadpoles derived from sympatry tend to grow faster—but metamorphose at smaller size—than those derived from allopatry (Pfennig and Pfennig 2005). Indeed, growth rate of tadpoles derived from at least one sympatric parent (i.e., SS, SA, AS) was greater than that for tadpoles in which both parents were derived from allopatry (see "Results"). Despite their sometimes faster growth rates, however, BCE offspring grew significantly less than WCE offspring, regardless of whether they were raised alone or with competitors, and regardless of which competitive environment their mother was derived from (Fig. 4).

Postmating isolation may evolve through two, nonmutually exclusive routes (Rice 1987; Hatfield and Schluter 1999; Rundle 2002). The first—ecologically dependent postmating isolation—occurs when "hybrids" (whether hybridization is between populations or species) are at a competitive disadvantage because they are intermediate in phenotype when an intermediate resource is lacking (Rice 1987; Hatfield and Schluter 1999; Rundle 2002). The second type—intrinsic postmating isolation—occurs when hybrids have reduced fitness for reasons unrelated to their environment; for example, due to genetic incompatibilities between parental genomes (reviewed in Coyne and Orr 2004).

Two lines of evidence suggest that ecologically dependent postmating isolation may be operating in this system. First, in terms of body size, the difference in performance of BCE versus WCE offspring was greater when each was raised with competitors as triplets versus when each was raised alone as singletons (Fig. 4A, B). Moreover, for BCE offspring of allopatric females, the difference in overall growth (increase in mass) and in growth rate of BCE versus WCE offspring was greater when each was

raised with competitors as triplets versus when each was raised alone as singletons (Fig. 4C, E; note, however, that frequency-dependent effects cannot be ruled out as a possible contributor to these results, given a lack of control for tadpole density between the triplet and singleton treatments). Second, at least some of the BCE offspring produced an intermediate phenotype that may have made them less effective at resource competition. In particular, AS offspring were significantly less carnivore-like in morphology than were AA offspring, even when reared under common conditions (Fig. 3A). Consequently, AS individuals were likely less effective than AA individuals at preying on the superior shrimp resource, just as we had predicted (see "Specific predictions").

We also found evidence of intrinsic incompatibilities. Specifically, BCE tadpoles grew less and were smaller than WCE tadpoles when reared alone. This finding raises the intriguing possibility that genetic incompatibilities may have evolved between populations that are only 3–11 km apart (Fig. 2A). Ecological selection pressures acting through character displacement may have promoted the evolution of these intrinsic genetic incompatibilities. In particular, because ecological selection acting against BCE offspring should lead to reduced gene flow between sympatric and allopatric environments, isolated populations in these divergent environments may have accumulated alleles that are incompatible in genomes from the alternative environment (reviewed in Coyne and Orr 2004). It is also possible that genetic divergence under character displacement has incidentally resulted in the evolution of intrinsic hybrid viability through pleiotropy or linkage (e.g., see Funk 1998).

Although our data are consistent with the hypothesis that character displacement has promoted the evolution of postmating isolation between *S. multiplicata* populations in contrasting competitive environments, the reduced performance of BCE offspring could have arisen because of selection imposed by agents other than interspecific competition. Ideally, to establish that interspecific competition per se has promoted the evolution of postmating isolation, such a study would be replicated in different geographical regions. Finding that WCE offspring consistently outperform BCE offspring in numerous, evolutionarily independent populations would greatly strengthen the case that character displacement has promoted the evolution of postmating isolation.

Given the reduced performance of BCE offspring, will populations in contrasting competitive environments eventually undergo speciation? Although sympatric and allopatric populations of *S. multiplicata* are potentially near to each other (3–11 km apart; Fig. 2A), there is very little (if any) ongoing gene flow between them (Rice and Pfennig, unpubl. data). Low levels of gene flow, coupled with strong divergent selection acting in different populations, increase the likelihood that speciation will occur (Mayr 1963; Schluter 2001; Coyne and Orr 2004). Our discovery of some level of postmating isolation between *S. multiplicata* populations

in contrasting competitive environments suggests that even if few individuals migrate between populations, those that do may have unfit offspring, further contributing to reduced gene flow.

Our results therefore reveal how postmating isolation between conspecific populations, and possibly even speciation, may emerge as a by-product of interactions between species. Although competitive interactions are likely to be important agents of divergent selection in this process (Schluter 2000; Rundle and Nosil 2005), interactions during mating (resulting in reproductive character displacement) may also promote divergence and, possibly, reproductive isolation between conspecific populations in sympatry with a heterospecific and those in allopatry (Hoskin et al. 2005; Jaenike et al. 2006; Pfennig and Ryan 2006). Thus, postmating isolation between conspecific populations, and possibly even speciation, may arise as an indirect consequence of selection for divergence between species during interspecific character displacement.

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