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EVOLUTION OF CHARACTER DISPLACEMENT IN SPADEFOOT TOADS: DIFFERENT PROXIMATE MECHANISMS IN DIFFERENT SPECIES

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Character displacement occurs when two species compete, and those individuals most dissimilar from the average resource-use phenotypes of the other species are selectively favored. Few studies have explored the sequence of events by which such divergence comes about. We addressed this issue by studying two species of spadefoot toads that have undergone ecological character displacement with each other. Previous research revealed that phenotypic shifts between sympatric and allopatric populations of one species, *Spea multiplicata*, reflect a condition-dependent maternal effect. Here, we show that analogous shifts in the other species, *S. bombifrons*, cannot similarly be explained by such a maternal effect, and that these shifts instead appear to be underlain by allelic differences. We hypothesize that these two species have evolved different mechanisms of character displacement because they differ in duration in sympatry. Specifically, because they occur at the edge of a range expansion, populations of *S. bombifrons* have been exposed to *S. multiplicata* for a longer period. Consequently, *S. bombifrons* have likely had more time to accumulate genetic changes that promote character displacement. Generally, character displacement may often progress through an initial phase in which trait differences are environmentally induced to one in which they are constitutively expressed.

KEY WORDS: **Divergent evolution, ecological character displacement, genetic assimilation, local adaptation, loss of plasticity, phenotypic plasticity.**

Ecological character displacement—trait evolution stemming from selection to lessen competition—has long been regarded as a major cause of species differences, speciation, and adaptive radiation (Brown and Wilson 1956; Grant 1972; Schluter 2000; Dayan and Simberloff 2005; Pfennig and Pfennig 2009). Yet, relatively little is known about the proximate mechanisms that mediate such trait evolution. Identifying the genetic and developmental bases of character displacement is crucial, however, because different proximate mechanisms can influence the potential speed of character displacement and, therefore, whether character displacement, as opposed to competitive exclusion, occurs (Pfennig and Pfennig 2009).

Generally, character displacement is assumed to arise when divergent selection imposed by another species causes changes in allele frequencies that ultimately generate shifts in traits associated with resource use (Grant 1972; Arthur 1982; Schluter and McPhail 1992; Taper and Case 1992; Schluter 2000). However, few attempts have been made to validate this critical supposition [for exceptions, see research on stickleback fish (Rundle and Schluter 2004) and Darwin's finches (Grant and Grant 2008)].

Indeed, rather than reflecting allelic differences, phenotypic shifts between competitors might be entirely environmentally induced; that is, arise via phenotypic plasticity (reviewed in Pfennig and Pfennig 2009). Many organisms respond adaptively to heterospecifics by facultatively expressing an alternative phenotype that lessens competitive interactions (Werner and Hall 1976; Robinson and Wilson 1994; Agrawal 2001; Pfennig and Murphy 2002; Fordyce 2006; Pfennig et al. 2006). Furthermore, some forms of plasticity—specifically, maternal effects (sensu Mousseau and Fox 1998)—can be transmitted reliably between generations (Agrawal et al. 1999; Plaistow et al. 2006; Allen et al. 2008; Pfennig and Martin 2009) and thereby form the basis of an alternative inheritance system by which adaptive evolution, such as character displacement, can unfold (Pfennig and Martin 2009).

One corollary of the assumption that character displacement stems from allelic differences is the further supposition that character displacement evolves using similar mechanisms in the interacting species. Yet, this need not be the case. Instead, two species involved in the same instance of character displacement might use different mechanisms to promote divergence in resource-use traits. In particular, the mechanism that mediates the shift from allopatric to sympatric populations of one species might differ from the mechanism that mediates the analogous shift in the other species (Fig. 1). Species might differ in mechanisms of character displacement for many reasons, such as if the species differ in: (1) relative population size (which may subsequently render genetic variation in one species more prone to random loss through genetic drift); (2) the intensity of selection favoring character displacement; (3) the genetic architecture underlying resource-use trait production; (4) levels of standing genetic variation; or (5) the length of time during which populations of each species have been in contact with the other species. For example, populations that have only recently confronted a heterospecific might be more prone to use phenotypic plasticity to diverge from their competitor in resource use. By contrast, populations that have had relatively long evolutionary contact with another species might have more time to evolve genetically fixed differences, possibly through the process of genetic assimilation, in which an environmentally induced trait (such as a divergent phenotype) no longer requires the original environmental stimulus to produce it and instead becomes an inherited, constitutively expressed trait (Waddington 1953; Lande 2009).

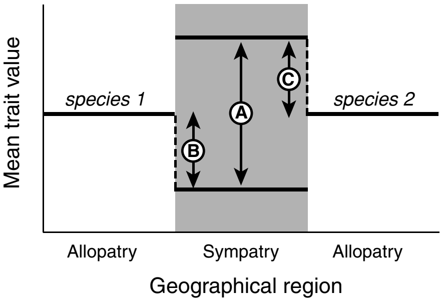

Figure 1. **Two species involved in the same instance of character displacement may use different mechanisms of divergence. Character displacement produces a distinctive pattern in which species differ more in sympatry than in allopatry [indicated by arrow (A)]. Divergence between species arises when, within each species, populations in sympatry diverge from those in allopatry [indicated by arrows (B) and (C)]. The mechanism mediating the phenotypic shift between such populations in one species may differ from that for the other species.**

Studies of interacting species that use different proximate mechanisms to promote the same instance of character displacement might be especially valuable in helping to clarify how the process of character displacement unfolds. Generally, little is known about the sequence of events that leads to character displacement. Here, we sought to explore these ideas empirically. We specifically investigated the proximate basis of character displacement in a species for which previous research indicates has undergone ecological character displacement with a heterospecific competitor. We also explored the proximate basis of character displacement between these two competing species. Finally, we asked whether each species possesses the same, or different, mechanisms of character displacement. We begin by describing the study system.

STUDY SYSTEM

Plains spadefoot toads, *Spea bombifrons*, and Mexican spadefoot toads, *S. multiplicata*, co-occur in the southwestern USA (Fig. 2). In the San Simon Valley of southeastern Arizona and southwestern New Mexico, these species have undergone character displacement with each other (Pfennig and Murphy 2000, 2002,

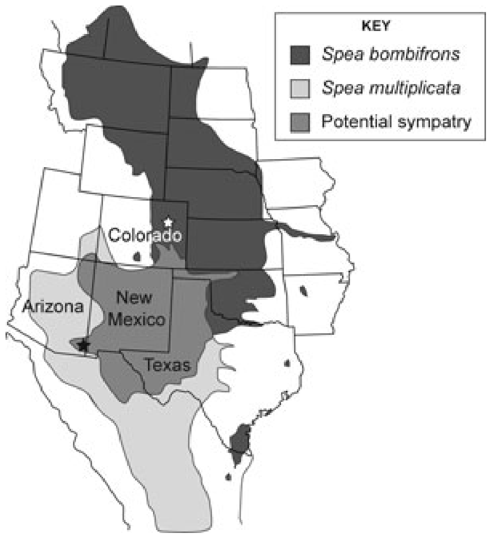

Figure 2. **Geographical ranges of *Spea bombifrons* and *S. multiplicata*, showing approximate locations from which study subjects were collected (dark star: sympatry; light star: allopatry).**

2003; Pfennig et al. 2006, 2007; Rice et al. 2009). In populations in which each species occurs alone, both species produce similar, intermediate frequencies of two ecomorphs: a large-headed carnivore morph, which prey on fairy shrimp, and a small-headed omnivore morph, which specializes on detritus (Pfennig et al. 2006). However, in populations in which they co-occur, *S. multiplicata* shift to producing mostly omnivores, whereas *S. bombifrons* shift to producing mostly carnivores (Pfennig and Murphy 2000, 2002, 2003; Pfennig et al. 2006). This divergence appears to reflect selection to lessen interspecific competition for food (Pfennig and Murphy 2000, 2002; Pfennig et al. 2007; Rice et al. 2009).

Although these differences between sympatric and allopatric populations of *S. multiplicata* persist even when tadpoles are reared under common conditions (Pfennig and Murphy 2000, 2002), they appear to be mediated by a condition-dependent maternal effect (Pfennig and Martin 2009). Specifically, in sympatry, *S. multiplicata* are forced by *S. bombifrons* onto the less-profitable detritus resource (Pfennig and Murphy 2000). As a result, *S. multiplicata* tend to express the smaller omnivore ecomorph (the larger carnivore ecomorph is triggered by shrimp ingestion; Pfennig 1990). Consequently, female *S. multiplicata* in sympatry mature as smaller (Pfennig and Pfennig 2005) and poorer condition adults (maternal "condition" is operationally defined here as the residual of female mass regressed against snout-vent length; e.g., see Pfennig 2007 and Pfennig and Martin 2009). Presumably because of their reduced size and/or condition, female *S. multiplicata* in sympatry invest less into offspring than do female *S. multiplicata* in allopatry (Pfennig and Martin 2009). Because smaller, poorer condition females produce mostly omnivores (Pfennig and Martin 2009; Martin and Pfennig 2010), these females create offspring with a phenotype that minimizes competition with *S. bombifrons* and that differs from the phenotype in allopatry. However, these population differences in morph production disappear once mothers are equilibrated in body condition (Pfennig and Martin 2009). Thus, a condition-dependent maternal effect mediates character displacement in *S. multiplicata*.

In contrast to *S. multiplicata*, the proximate basis of character displacement in *S. bombifrons* is unknown, as is the proximate basis of the observed differences between species in morph production (see above). We therefore investigated the proximate basis of: (1) character shifts between sympatric and allopatric populations of *S. bombifrons*; and (2) character displacement between these two species.

Materials and Methods

PROXIMATE BASIS OF POPULATION DIFFERENCES

We performed an experiment to determine whether *S. bombifrons* sibships derived from sympatry differ in morph production from sibships derived from allopatry, even when tadpoles are reared under common conditions. We also sought to determine if any such differences in morph production reflect differences between populations in maternal size or condition, as has been found in *S. bombifrons*' competitor, *S. multiplicata* (see section Study System).

We began by collecting adult *S. bombifrons* at multiple allopatric and sympatric sites. Allopatric sites were near Last Chance, Washington County, Colorado USA (about 130 km north of the most northerly populations of *S. multiplicata*; Hammerson 1999). Sympatric sites were in the San Simon Valley of Cochise County, Arizona and adjacent Hidalgo County, New Mexico USA, where character displacement has been documented between the two species (Pfennig and Murphy 2000, 2002, 2003; Pfennig et al. 2006, 2007). Adults were transported to the University of North Carolina, where they were used in the experiment below.

To initiate the experiment, we paired 13 females and 13 males that had been collected from multiple populations near Last Chance (allopatry) and nine females and nine males that had been collected from multiple populations in the San Simon Valley (sympatry). Immediately before breeding, we measured each individual's snout-vent length (SVL) and mass (these data were used to calculate maternal condition; see section Study System). We then injected both females and males with 0.07 mL of 0.1 mM gonadotropin releasing hormone agonist and placed each male–female pair in an 11.3 L tank filled with dechlorinated water overnight. From these breedings, we obtained 13 allopatric sibships and nine sympatric sibships of tadpoles.

Three days after the tadpoles hatched, we randomly selected 30 similarly sized tadpoles from each sibship and placed them in pairs in replicate tanks (28 × 18 × 10 cm) filled with 6 L of dechlorinated water. We fed tadpoles live brine shrimp (*Artemia* sp.) ad libitum (brine shrimp are similar to the fairy shrimp on which *Spea* tadpoles feed in natural ponds and can induce carnivore-like morphology; Pfennig et al. 2007). After 10 days, we categorized each tadpole as an omnivore or a carnivore using criteria in Pfennig (1990). Using these data, we estimated each sibship's probability of producing a carnivore by identifying the number of replicate tanks that produced at least one carnivore out of the total number of replicate tanks for each sibship. We then killed each tadpole by immersion in a 0.1% aqueous solution of tricane methanesulfonate (MS 222) and measured its mass and orbitohyoideus muscle (OH) width. OH width, corrected for tadpole body size (mass), provided a continuous measure of trophic phenotype, with larger values indicative of more carnivore-like tadpoles (Pfennig 1990).

The two response measures used for this experiment (probability of sibships producing a carnivore and size-adjusted OH width) differed from the response measure used in earlier studies of character displacement in the congener, *S. multiplicata*, where shrimp eating time served as a proxy for carnivore production (Pfennig and Martin 2009; Martin and Pfennig 2010). However, a sibship's mean shrimp-eating time is highly significantly correlated with its propensity to produce carnivores (see Pfennig et al. 2007). Thus, the response measures used here are directly comparable to those used earlier.

To determine if sibships whose parents were derived from allopatry differed in larval phenotype from sibships whose parents were derived from sympatry, we first analyzed the probability of producing a carnivore with a generalized linear model (GLM). Because we scored each sibship's probability of producing a carnivore as a binary response (each replicate tank was scored as either containing at least one carnivore or alternatively, containing only omnivores), we used a binomial distribution with a probit link function. We then evaluated all combination of models with the following parameters: (1) source population (i.e., sympatry or allopatry); (2) maternal body size (SVL); (3) paternal body size (SVL); (4) maternal condition (the interaction between maternal mass and SVL); and (5) all interactions between parental size/condition and source population. We evaluated the possible effect of maternal condition on offspring phenotype, because previous studies revealed that maternal condition influences offspring morph determination in the closely related species, *S. multiplicata* (Pfennig and Martin 2009). Moreover, we evaluated the possible effect of parental SVL on offspring phenotype, because previous studies revealed that maternal body size also influences offspring morph determination in *S. multiplicata* (Martin and Pfennig 2010). We then compared models using Akaike's information criteria corrected for sample size (AICc) following Burnham and Anderson (2002). Specifically, we chose the model with the lowest AICc score. Because the four top models did not differ by ΔAICc > 2, we chose the model with fewer parameters. We statistically evaluated the chosen model with an analysis of deviance (Table 1).

We next evaluated if OH width differed between sibships derived from sympatric versus allopatric populations. To do so, we fit a linear mixed model with the random effect of tank, nested within the random effect of sibship, using restricted maximum likelihood (REML). We fit OH width as the response variable, with tadpole mass as a covariate (to correct OH width for differences in body size). Using AICc, we then compared models with all combinations of the five additional parameters described in the previous paragraph, as well as the interaction between source population and tadpole mass, to determine if the allometry between body size and OH width differed between source populations. We then chose the model with the lowest AICc score (ΔAICc $>$ 2). We statistically evaluated the effect of each parameter and interaction in the chosen model by comparing with an analysis of variance (ANOVA), the full model to a model with the parameter or interaction of interest removed (Table 2).

Additionally, we used separate linear models to contrast overall body size (SVL) and condition (using female mass as the response variable and female SVL and source population as covariates) of the sympatric females bred in the previous experiment to that of the allopatric females. All analyses were carried out using R statistical software (version 2.9.2; R Development Core Team).

PROXIMATE BASIS OF SPECIES DIFFERENCES

Because the present study revealed that phenotypic shifts between sympatric and allopatric populations of *S. bombifrons* could not be explained by a condition-dependent maternal effect (see section Results), we investigated the proximate mechanism of character displacement between *S. bombifrons* and *S. multiplicata*. To do so, we created hybrids between species and used the resource-use phenotype expressed by hybrid versus pure-species offspring to infer the proximate basis of trait differences between species.

We collected adults of both species from multiple sympatric sites in the San Simon Valley. We then bred these adults in two different years (using different adults each year) to create four cross-types: (1) *S. bombifrons* female × *S. bombifrons* male ("BB" cross-type); (2) *S. bombifrons* female × *S. multiplicata* male ("BM" cross-type); (3) *S. multiplicata* female × *S. bombifrons* male ("MB" cross-type); and (4) *S. multiplicata* female × *S. multiplicata* male ("MM" cross-type).

Table 1. **Results of the generalized linear model selected using AICc on the probability of sibships producing a carnivore (*PrC*). Shown is an analysis of deviance (Binomial, link: Probit) of *PrC*.**

Model: *PrC=population*

Term	β (SE)[1]	df	Deviance	Residual df	Residual deviance	P $[P(>\lvert\chi^2\rvert)]$
Null model	−0.89 (0.1)	21			28.59	
Population		1	6.85	20	21.74	0.009
Allopatry[2]						
Sympatry	0.4 (0.15)					

[1] β, linear parameter estimate; SE, standard error.

[2] Baseline level for treatment contrasts.

Table 2. **Results of the linear mixed model selected using AICc on size-standardized orbitohyoideus muscle width (*OH width*).**

Model: *OH width=tadpole mass+population+tadpole mass×population, random=(1|sibship*[1]*/tank*[2]*)*

Term	β (SE)[3]	df	*F*	*P*
Intercept	0.07 (0.03)	1, 310	6.11	0.014
Tadpole mass	−0.19 (0.04)	1, 310	4.76	0.029
Population		1, 20	0.344	0.344
Allopatry[4]				
Sympatry	−0.02 (0.04)			
Tadpole mass×population		1, 310	14.132	0.0002
Allopatry[4]				
Sympatry	0.33 (0.06)			

[1] Sibship accounts for 13.8% of the total variance.

[2] Tank accounts for 6.18% of the total variance.

[3] β, linear parameter estimate; SE, standard error.

[4] Baseline level for treatment contrasts.

For the first breeding, we created 21 sibships: five BB sibships, five BM sibships, five MB sibships, and six MM sibships. Three days after the tadpoles hatched, we randomly selected 20 similarly sized tadpoles from each sibship and placed them individually in replicate tanks (20 replicate tanks per sibship) and reared them as before. After 10 days, we categorized each tadpole as an omnivore or a carnivore and estimated each sibships' probability of producing a carnivore as in the previous experiment. As before, we used a GLM with a binomial distribution and a probit link function to determine if cross-types differed in probability of producing a carnivore. We used cross-type as our main effect (we did not include parental body size in this analysis, as these data were not available). Because the model revealed that there were significant differences among cross-types, we used Tukey–Kramer HSD tests to determine which groups differed.

For the second breeding, we created 27 new sibships: four BB sibships, four BM sibships, four MB sibships, and 15 MM sibships. Three days after the tadpoles hatched, we randomly selected similarly sized tadpoles from each sibship. Tadpoles were placed in pairs (two nonsiblings) in replicate tanks (28 × 18 × 10 cm) filled with 6 L of dechlorinated water such that there were 55 replicate tanks for the BB, BM, and MB cross-types and 223 replicate tanks for the MM cross-type (there were either 27 or 28 replicate tanks per sibship). We fed the tadpoles as before. After 12 days, the tadpoles were killed by immersion in a 0.1% aqueous solution of MS 222. We then measured the mass and orbitohyoideus muscle (OH) width of each tadpole. To determine if cross-types differed in OH width, we fit a linear mixed model to the data, with tank as a random effect, using REML, with OH width as the response variable and tadpole mass as a covariate. We then evaluated models with cross-type, tadpole mass, and the interaction between cross-type and tadpole mass, to determine if size-corrected OH width and the allometry between body size and OH width differed between cross-types. Including cross-type and the interaction between cross-type and tadpole mass was justified as it improved the AICc score of the model ((AICc of model with mass only) − (model including cross-type and mass × cross-type) = ΔAICc > 2). We did not include parental body size in this analysis, as these data were not available. Because the model revealed that there were significant differences among cross-types, we used Tukey–Kramer HSD tests to determine which groups differed in OH width, adjusted for body size. In addition, because the model revealed a significant interaction between cross-type and tadpole mass, we made pairwise comparisons of the relationship between OH width and tadpole mass for each cross-type to determine which groups differed in their allometry. All analyses were carried out using R statistical software (version 2.9.2; R Development Core Team).

For both breedings, we used the resource-use phenotype expressed by hybrid versus pure-species offspring to infer the nature of the genetic differences between species. Specifically, we predicted that if a maternal effect mediates differences between species, then hybrids should resemble the pure-species cross-type that shared the same mother; that is, the BM offspring should resemble BB offspring, and MB offspring should resemble MM offspring. By contrast, finding that hybrids were either intermediate between pure-species cross-types or identical to the same pure-species cross-types would point to genetically based species differences.

Results

PROXIMATE BASIS OF POPULATION DIFFERENCES

Sibships derived from sympatry differed in tadpole trophic phenotype from sibships derived from allopatry, even when reared under

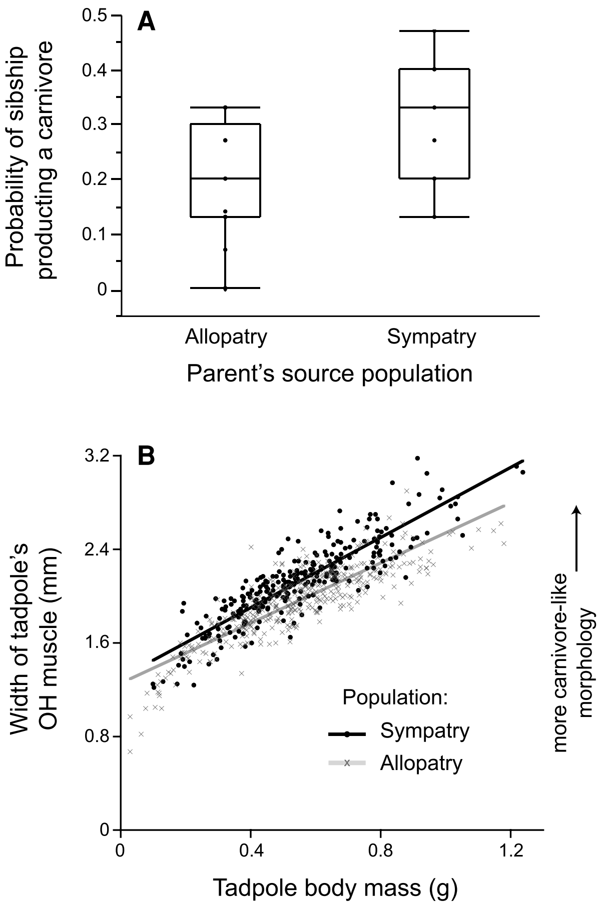

Figure 3. Sibships derived from sympatry differ in tadpole trophic phenotype from sibships derived from allopatry, even when reared under common conditions. (A) The probability of producing a carnivore for sibships (dots) from sympatry versus allopatry; box plots show 10th, 25th, 50th (median), 75th, and 90th percentiles. (B) Relationship between the width of the orbitohyoideus (OH) muscle (more carnivore-like tadpoles have larger muscles) and body size (mass) for individual tadpoles (dots) from sympatry versus allopatry.

common conditions (Fig. 3). Specifically, there was a significant effect of population (i.e., sympatry vs. allopatry) on the probability of sibships producing a carnivore (Table 1). As predicted (see section Study System), the mean (±SEM) probability of producing a carnivore was greater for sibships derived from sympatry (0.31 ± 0.04, $N = 9$ sibships) than for sibships derived from allopatry (0.20 ± 0.03, $N = 13$ sibships). Also as predicted, sibships derived from sympatry differed from sibships derived from allopatry in OH width. Although there was not a significant effect of source population on OH width alone, we found a significant interaction between the covariate of tadpole mass and population (i.e., sympatry vs. allopatry) on OH width (Table 2). Specifically, the slope of the interaction between OH width and tadpole mass was steeper for sibships derived from sympatry, suggesting differences in allometry for OH width, and therefore differences in trophic phenotype between populations (Fig. 3).

Differences in trophic phenotype between sympatric versus allopatric populations do not appear to reflect a condition-dependent maternal effect. In particular, models containing parental size, condition, or interactions of these parameters were not chosen as the top model in our model selection process. There were three additional models of the probability of producing a carnivore that did not differ from our chosen model by ΔAICc > 2. The three models were (1) $PrC = population + paternal\ SVL$; (2) $PrC = population + maternal\ SVL$; and (3) $PrC = population * paternal\ SVL$. However, these models were more complex than our chosen model. In addition, evaluating these models with analysis of deviance showed that only the term *population* had a significant effect in each model ($P < 0.05$). Moreover, separate linear models revealed that differences between sympatric and allopatric populations in morph production did not reflect variation between populations in either maternal condition (Population + Female SVL: $F_{1,19} = 0.004$, $P = 0.941$) or maternal size (SVL) (Population: $F_{1,20} = 0.118$, $P = 0.734$).

PROXIMATE BASIS OF SPECIES DIFFERENCES

Species cross-types differed in tadpole trophic phenotype, even when reared under common conditions. Specifically, there was a significant effect of cross-type (i.e., MM, BB, MB and BM) on both the probability of producing a carnivore (Table 3) and OH width (Table 4). Moreover, we found a significant interaction between the covariate of tadpole mass and cross-type on OH width (Table 4), suggesting differences in allometry for OH width between cross-types. Post hoc tests between all pairs of cross-types revealed that the allometry of OH width was steeper in hybrid cross-types than in either parental cross-type ($P < 0.05$). However, neither the hybrid cross-types nor the parental cross-types differed in their allometry ($P > 0.05$).

Differences between species in trophic phenotype do not appear to have been inherited maternally. For the probability of producing a carnivore (year 1 experiment), BB offspring (i.e., pure *S. bombifrons* offspring) did not differ significantly from either BM or MB offspring (i.e., the offspring of either hybrid cross-type; Tukey–Kramer HSD: $P > 0.05$; Fig. 4A). For OH width (year 2 experiment), MB offspring did not differ significantly from BB offspring (Tukey–Kramer HSD: $P > 0.05$; Fig. 4B), but BM offspring differed significantly from all other cross-types (Tukey–Kramer HSD: $P < 0.05$; Fig. 4B). However, for both years, and for both response measures, the offspring of all three cross-types in which at least one parent was *S. bombifrons* differed significantly from the offspring of the MM cross-type (i.e., pure *S. multiplicata* offspring; Tukey-Kramer HSD: $P < 0.05$; Fig. 4).

Table 3. Results of a generalized linear model of the effect of species cross-type on the probability of sibships producing a carnivore (*PrC*). Shown is an analysis of deviance (Binomial, link: Probit) of *PrC*.

Model: *PrC=population*

Term	β (SE)[1]	df	Deviance	Residual df	Residual deviance	*P* [$P(>\|\chi^2\|)$]
Null model	0.58 (0.27)	20			53.24	
Cross-type		3	32.85	17	20.39	<0.0001
BB[2]						
BM	0.12 (0.38)					
MB	0.26 (0.39)					
MM	−1.55 (0.38)					

[1] β, linear parameter estimate; SE, standard error.

[2] Baseline level for treatment contrasts.

Discussion

Although ecological character displacement has long been viewed as an important source of adaptive diversification, little is known about its proximate causes (Pfennig and Martin 2009). In this study, we explored the proximate basis of character displacement in spadefoot toads, *Spea bombifrons* and *S. multiplicata*. Previous research revealed that phenotypic shifts between sympatric and allopatric populations of *S. multiplicata* arise through a condition-dependent maternal effect (see **STUDY SYSTEM**). However, prior to this study, little was known about the proximate basis of the analogous character shifts in their competitor, *S. bombifrons*.

As in *S. multiplicata*, *S. bombifrons* derived from sympatry differed in morph production from *S. bombifrons* derived from allopatry, even when tadpoles from both regions were reared under common conditions (Fig. 3). Unlike the situation for *S. multiplicata*, however, these phenotypic shifts do not appear to be mediated by a condition-dependent maternal effect. In particular, in contrast to what was found in *S. multiplicata* (Pfennig and Martin 2009; Martin and Pfennig 2010), we found: (1) no significant effects of either maternal body size or maternal body condition in explaining differences in morph production between sympatric and allopatric populations; (2) no significant differences between sympatric and allopatric populations in either maternal body size or maternal body condition; and (3) no significant relationship between variation among sibships in the probability of producing a carnivore and either maternal body size or maternal body

Table 4. Results of the linear mixed model evaluating the effect of species cross-type on size-standardized orbitohyoideus muscle width (*OH width*).

Model: *OH width=tadpole mass+cross-type+tadpole mass×cross-type, random=(1|tank[1])*

Term	β (SE)[2]	df	*F*	*P*
Intercept	1.25 (0.05)	1, 270	28877.13	<0.0001
Tadpole mass	1.22 (0.09)	1, 240	1801.33	<0.0001
Cross-type		1, 3	43.282	<0.0001
BB[3]				
BM	−0.22 (0.07)			
MB	−0.21 (.07)			
MM	−0.363 (0.06)			
Tadpole mass × cross-type		1, 240	4.2	0.063
BB[3]				
BM	0.25 (0.12)			
MB	0.43 (0.12)			
MM	0.19 (0.12)			

[1] Tank accounts for ∼0% of the total variance.

[2] β, linear parameter estimate; SE, standard error.

[3] Baseline level for treatment contrasts.

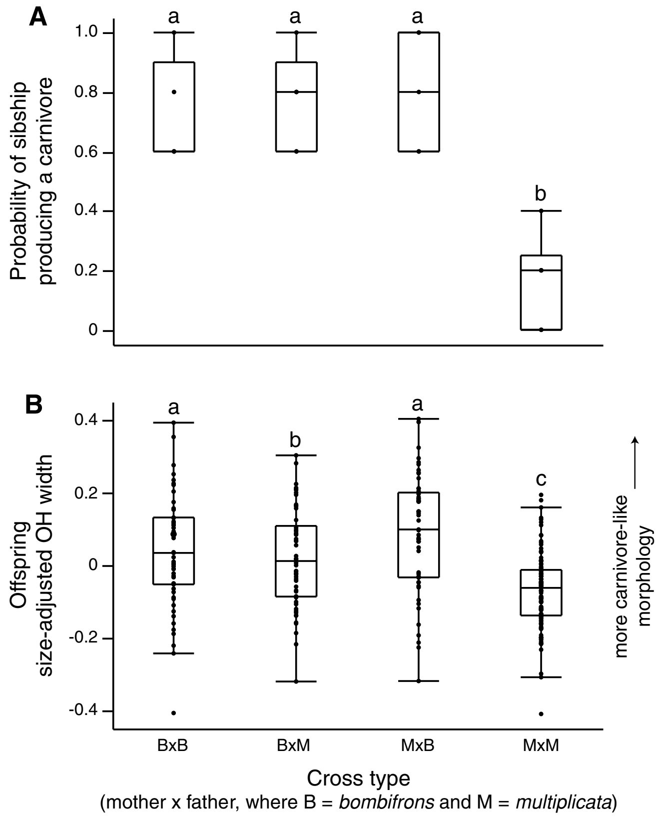

Figure 4. Comparisons among cross-types in (A) the probability of sibships producing a carnivore, and (B) size-adjusted OH width. Dots are values for (A) sibships or (B) individual tadpoles. Box plots show 10th, 25th, 50th (median), 75th, and 90th percentiles; cross-types not connected by the same letter are significantly different from one another.

condition. Therefore, differences in morph production between sympatric and allopatric populations of *S. bombifrons* (a manifestation of character displacement) do not appear to reflect a condition-dependent maternal effect. Instead, these canalized shifts appear to reflect underlying genetic differences between sympatry and allopatry.

This study also sheds light on the proximate basis of the character displacement between *S. bombifrons* and *S. multiplicata*. In particular, based on the phenotypes produced by the hybrids (Fig. 4), a maternal effect is an unlikely explanation for the differences between species in morph expression. Instead, these data suggest that phenotypic differences between species reflect genetic differences.

The most surprising outcome from these experiments was finding that these two species differ in a proximate mechanism of character displacement. In particular, whereas character shifts between sympatric and allopatric populations of *S. multiplicata* are mediated by a condition-dependent maternal effect (Pfennig and Martin 2009), the analogous shifts in *S. bombifrons* cannot similarly be explained by such a maternal effect. Instead, character displacement in *S. bombifrons* appears to reflect genetic differences between populations (see above). This result was unexpected, because these two species are involved in the same instance of character displacement, and they are similar ecologically and phylogenetically. However, as noted in the Introduction, species might often differ in the proximate basis of character displacement for a number of reasons, including differences in length of time during which populations of each species have been in contact with the other species.

Generally, populations that have only recently confronted a heterospecific competitor might undergo environmentally induced divergence in resource use. Because these responses can arise as soon as a population encounters a heterospecific competitor (e.g., see Pfennig and Murphy 2000), such plasticity may buffer populations from extinction (via competitive exclusion) while genetic changes accumulate that produce the divergent trait constitutively. Indeed, populations that have had relatively long evolutionary contact with another species might be more prone to evolve such genetically fixed differences. They too might have initially expressed character displacement through environmentally induced phenotypic shifts, but, over time, these shifts might have been reinforced by allelic differences.

In the present case, sympatric populations of each species do appear to differ in how long they have been in contact with the other species. In particular, population genetic surveys (Rice and Pfennig 2008) indicate that *S. bombifrons* has undergone a widespread range expansion out of its ancestral range in the southern Great Plains, and that this species is a recent invader into the San Simon Valley (Fig. 2), where *S. multiplicata* was already resident (in contrast to *S. bombifrons*, *S. multiplicata*'s range appears to have been stable; Rice and Pfennig 2008). Because *S. bombifrons* has apparently been expanding its range southwestward, thereby continually invading new habitat formerly occupied solely by *S. multiplicata* (Fig. 2), populations of *S. bombifrons* on the wave front of the expansion (e.g., in the San Simon Valley) have likely had long evolutionary contact with *S. multiplicata*. Consequently, this species has likely had more time to accumulate genetic changes that promote character displacement. By contrast, populations of *S. multiplicata* along this wave front have likely only recently encountered *S. bombifrons*. Indeed, *S. bombifrons* has recently increased in frequency in this area (Pfennig 2003), suggesting that *S. multiplicata* may have only recently experienced significant competitive pressures from *S. bombifrons*. This might explain why character displacement in *S. multiplicata* from the San Simon Valley is mediated by a condition-dependent maternal effect (Pfennig and Martin 2009): these populations may not have had sufficient time to accumulate genetic changes that produce divergent traits.

As in present-day *S. multiplicata* in the San Simon Valley, character shifts in ancestral sympatric *S. bombifrons* may have gone through an initial evolutionary phase in which they were environmentally induced (a manifestation of "facultative character displacement"; sensu Pfennig and Murphy 2002). Such environmentally induced shifts in trait expression would have enabled ancestral *S. bombifrons* to respond rapidly—and adaptively—to competition from *S. multiplicata* by producing carnivores only (the shift to producing only carnivores, and no omnivores, would have been adaptive for *S. bombifrons*, because *S. multiplicata* are the superior competitor for the omnivore's resource of detritus; Pfennig and Murphy 2000). It is unknown, however, whether ancestral sympatric *S. bombifrons* then went through an evolutionary phase in which character shifts were mediated by a condition-dependent maternal effect (as also in present-day *S. multiplicata* in the San Simon Valley; Pfennig and Martin 2009). One might expect that *S. bombifrons* would have gone through such a phase, given that facultative character displacement can greatly influence maternal size and condition (Pfennig and Pfennig 2005), which can in turn bias offspring phenotype (Pfennig and Martin 2009; Martin and Pfennig 2010). Nevertheless, no vestiges of such a condition-dependent maternal effect were detected among the *S. bombifrons* in this study (e.g., female *S. bombifrons* from sympatry did not differ from female *S. bombifrons* in allopatry in either body size or condition, as might be expected had they formerly gone through such a "maternal-effect phase").

Once species (and, within each species, sympatric and allopatric populations) diverge, phenotypic plasticity might bring about more permanent divergence by promoting the differential fixation of alternative phenotypes in populations that no longer experience both environments. The loss of plasticity—and the subsequent fixation of the newly favored, divergent phenotype—occurs through genetic assimilation (Waddington 1953) and can proceed via two routes. First, when the maintenance or expression of plasticity is costly (Relyea 2002), selection may actively eliminate plasticity and instead favor alleles that regulate expression of the newly favored, divergent trait (West-Eberhard 2003). This process could occur through either the selective sorting of standing variation (Barrett and Schluter 2008) and/or genetic accommodation (sensu West-Eberhard 2003). Second, plasticity can be lost through mutational degradation or genetic drift (Masel et al. 2007). Experiments have demonstrated the loss of plasticity (Suzuki and Nijhout 2006), and data from natural populations suggest that the resulting differential fixation of alternative phenotypes in different populations can drive divergence between populations and species (Badyaev et al. 2002; Pfennig and Murphy 2002). Thus, environmentally induced phenotypic change might have preceded—and even facilitated—the evolution of genetic differences that stabilized phenotypic differences between species and populations experiencing divergent selection.

The above scenario for how character displacement might unfold—from an initial evolutionary phase in which divergence is environmentally induced to one in which divergence is underlain by allelic differences—is likely not unique to spadefoots. For example, in certain small coastal lakes of southwestern Canada, two species of sticklebacks of the *Gasterosteus aculeatus* complex occur together, one of which expresses a distinctive "benthic" phenotype, and the other of which expresses a distinctive "limnetic" phenotypic. These same two ecotypes have evolved repeatedly, and independently, in different lakes (reviewed in Rundle and Schluter 2004), indicating that ancestral sticklebacks evolved parallel phenotypic variation as they adapted independently to similar selective pressures; in this case, costly ecological and reproductive interactions with a phenotypically similar heterospecific. Such repeated episodes of character displacement are thought to have arisen following the invasion from the marine environment by an ancestral limnetic ecotype into a lake already occupied by a solitary, intermediate ecotype. As a consequence of character displacement, a new benthic ecotype arose within each such lake, replacing the ancestral, intermediate ecotype (reviewed in Rundle and Schluter 2004).

The early stages of character displacement in sticklebacks appear to have been mediated by phenotypic plasticity. In particular, experiments have revealed that diet-induced plasticity in marine stickleback (representing the ancestral colonists) produces phenotypes similar to those expressed in derived freshwater benthic and limnetic ecotypes (Wund et al. 2008). In present-day populations, however, phenotypic differences between benthic and limnetic ecotypes reflect genetic differences (reviewed in Rundle and Schluter 2004). Thus, as in spadefoots, character displacement in sticklebacks may have unfolded as it transitioned from an initial phase in which trait divergence was environmentally induced to one in which such divergence became expressed constitutively.

Ancestral plasticity has also been implicated in character displacement in other systems, such as *Anolis* lizards (Losos et al. 2000) and numerous species of freshwater fish (Robinson and Wilson 1994), suggesting that character displacement might often progress through an initial phase in which trait divergence is environmentally induced to one in which such divergence is expressed constitutively (e.g., see Wilson 1992, p. 174). However, further research is needed to evaluate the validity and generality of this model. Indeed, little is known about how character displacement progresses through the course of its evolution.

In sum, this study illustrates how two species involved in the same instance of character displacement can use different mechanisms to promote divergence in resource-use traits. Species pairs undergoing character displacement might often differ in mechanism if they differ in how long they have been in contact with each other. Such systems may be especially valuable for shedding light onto the sequence of events by which character displacement unfolds.

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