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Reinforcement generates reproductive isolation between neighbouring conspecific populations of spadefoot toads

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Reproductive character displacement is the adaptive evolution of traits that minimize deleterious reproductive interactions between species. When arising from selection to avoid hybridization, this process is referred to as reinforcement. Reproductive character displacement generates divergence not only between interacting species, but also between conspecific populations that are sympatric with heterospecifics versus those that are allopatric. Consequently, such conspecific populations can become reproductively isolated. We compared female mate preferences in, and evaluated gene flow between, neighbouring populations of spadefoot toads that did and did not occur with heterospecifics (mixed- and pure-species populations, respectively). We found that in mixed-species populations females significantly preferred conspecifics. Such females also tended to prefer a conspecific call character that was dissimilar from heterospecifics. By contrast, females from pure-species populations did not discriminate conspecific from heterospecific calls. They also preferred a more exaggerated conspecific call character that resembles heterospecific males. Moreover, gene flow was significantly reduced between mixed- and pure-species population types. Thus, character displacement (and, more specifically, reinforcement) may initiate reproductive isolation between conspecific populations that differ in interactions with heterospecifics.

1. Introduction

Reproductive character displacement is the process by which traits evolve in response to selection to minimize deleterious reproductive interactions with heterospecifics [1]. When stemming from selection to avoid hybridization, this process is also known as ‘reinforcement’ [1–3], and we therefore use the term ‘reproductive character displacement’ to include reinforcement. Generally, such trait evolution promotes divergence between sympatric species in reproductive traits and can therefore contribute to the completion of the speciation process [1,3–6].

Most research on reproductive character displacement—especially reinforcement—has focused on its role in completing speciation [1,4,5]. Yet character displacement can also *initiate* speciation [1,2,6–17]. In particular, because selection to avoid heterospecifics acts only where heterospecifics are actually encountered, traits that evolve via reproductive character displacement in sympatry do not also evolve in allopatric populations. If such trait differences become sufficiently pronounced, individuals in sympatry or allopatry may fail to recognize members of the alternative population type as acceptable mates [8,11,12,17]. Conspecific populations in sympatry and allopatry could thereby become reproductively isolated, and, ultimately, speciation between them could occur [1,2,6–17] (but see [18,19]).

If and when reinforcement initiates divergence between sympatry and allopatry remains an open area of inquiry [1,2,7,9,14,20]. Whether conspecific populations in sympatry versus allopatry become divergent to the point of being reproductively isolated depends on at least two key factors. First, if gene flow between sympatry and allopatry is already low or absent (e.g. owing to distance or physical barriers), then such populations are more likely to evolve local adaptations to the presence or absence of heterospecifics that can further promote reproductive isolation [14,18,20]. Indeed, character displacement is most likely to

generate reproductive isolation between conspecific populations in sympatry and allopatry when such populations are physically isolated (by long distances or geographical barriers) or occur in patchy habitats [14,18,20]. Whether such divergence can occur among neighbouring populations within contiguous habitat that potentially experience gene flow remains an open question [14,18,20].

Second, whether character displacement drives divergence between allopatry and sympatry depends on its effect on mating behaviour [1,2,7–10,13,21,22]. Generally, reproductive character displacement is expected to promote the evolution of female mate preferences that minimize interactions with heterospecifics. If the evolution of such preferences concomitantly alters female preferences for conspecific males, then reproductive isolation between conspecific populations in sympatry and allopatry become plausible. Indeed, if, as a consequence of reproductive character displacement, sympatric females find allopatric conspecific males unattractive and allopatric females find sympatric conspecific males unattractive, then reproductive isolation between sympatry and allopatry becomes likely [1,2,7,21,23–26]. Such divergent mating behaviour can occur when reproductive character displacement generates trade-offs in optimal patterns of either female preference or male trait expression in sympatric versus allopatric populations [1,2].

We sought to evaluate whether reproductive character displacement can promote divergence in mating behaviours and, consequently, reproductive isolation between adjacent sympatric and allopatric populations that potentially experience gene flow. We used spadefoot toads, *Spea multiplicata*, as our study system. Spadefoots are explosive breeders in which females select males based on aspects of their mating calls [23,27].

Spea multiplicata hybridizes with a congener, *Spea bombifrons*, where they co-occur in the southwestern USA and northwestern Mexico [28–32]. For *S. multiplicata* (but not *S. bombifrons*), hybridization is always costly, and female *S. multiplicata* are therefore under strong selection to avoid hybridization [31,33]. Consequently, sympatric females have evolved preferences for conspecific males with slower call rates (to avoid the faster-calling *S. bombifrons*), whereas allopatric *S. multiplicata* from outside of *S. bombifrons*'s geographical range prefer conspecifics with faster call rates [23].

Faster call rates are potentially indicative of male condition [34,35] and his ability to confer fitness benefits to females or their offspring. Indeed, allopatric females obtain good-condition mates that provide both enhanced fertilization success and better-quality offspring, whereas sympatric females do not [23,24]. Nevertheless, preferences for slower call rates by sympatric females contribute to hybridization avoidance [23]. Indeed, in populations in southeastern Arizona, USA, hybridization has declined over time [32,36], as is expected when reinforcement occurs [36–40].

Although the ranges of *S. multiplicata* and *S. bombifrons* overlap over a large region, populations at a local scale consist of either a single species or both species. Moreover, whether a given population consists of a single species or both species appears stable over time (K. Pfennig 1995–2013, personal observation and unpublished data). Neighbouring *S. multiplicata* populations—between which these toads potentially migrate—therefore differ in interactions with heterospecifics. Consequently, populations of *S. multiplicata* that differ in the presence of *S. bombifrons* could undergo divergent evolutionary trajectories in the evolution of reproductive traits and thereby become reproductively isolated.

To address this possibility, we evaluated female *S. multiplicata* mate preferences in, and gene flow between, populations that do and do not co-occur with *S. bombifrons* within the San Simon Valley in southeastern Arizona (figure 1). Within this region, both *S. multiplicata* and *S. bombifrons* co-occur and have historically hybridized in low-elevation populations (hereafter 'mixed-species' populations). As elevation increases, however, populations consist of only *S. multiplicata* (hereafter 'pure-species' populations).

Ecological factors other than the presence of *S. bombifrons* could be associated with elevation, and thereby potentially contribute to divergence between pure- and mixed-species populations of *S. multiplicata*. Yet the desert-scrub habitat in which pure- and mixed-species populations occur is similar and contiguous. Instead, interactions between the two species, rather than elevation-dependent environmental variables, appear to determine whether or not *S. multiplicata* and *S. bombifrons* co-occur at the local scale [21,41]. In particular, the relative costs and benefits of hybridization for *S. bombifrons*, which sometimes benefits by hybridizing with *S. multiplicata*, play a potential role in co-occurrence [42] (K. Pfennig 2004–2013, unpublished data). Moreover, resource competition between *S. multiplicata* and *S. bombifrons* at the tadpole stage appears to be a dominant factor that affects patterns of co-occurrence at the local scale [41]. Indeed, ecological character displacement driven by resource competition has contributed to divergent resource-use morphologies in the tadpoles ([43] and references therein)—and reduced gene flow [44]—between pure- and mixed-species populations.

Although ecological character displacement contributes to divergence between pure- and mixed-species populations of *S. multiplicata*, these populations could also diverge in mating behaviour owing to differences in risk of hybridizing with *S. bombifrons*. Indeed, the frequency of *S. bombifrons* *per se* appears to be a better predictor of patterns of sexual selection driven by mate choice than is elevation [21]. This pattern is not expected if some other environmental factor associated with elevation drives divergence in mating behaviour. Such a pattern is also not fully explained by ecological character displacement driving divergence between pure- and mixed-species populations of *S. multiplicata*.

Our specific goals were twofold. First, we evaluated female mate preferences in neighbouring populations of *S. multiplicata* that differed in the presence of *S. bombifrons*. We expected that reinforcement in mixed-species populations should promote divergence in mate preferences between pure- and mixed-species populations of *S. multiplicata*. Second, we measured population structure to determine whether gene flow is reduced between *S. multiplicata* populations experiencing *different* selective environments (i.e. pure- versus mixed-species populations) relative to that between populations within the *same* selective environment. Finding such reduced gene flow would suggest that reinforcement—and, more generally, reproductive character displacement—can promote reproductive isolation between neighbouring conspecific populations.

2. Material and methods

(a) Female mate preferences

Females used in the preference tests were wild caught as reproductively mature adults in populations near Portal, Arizona, and Rodeo, New Mexico, USA. Females were either caught while

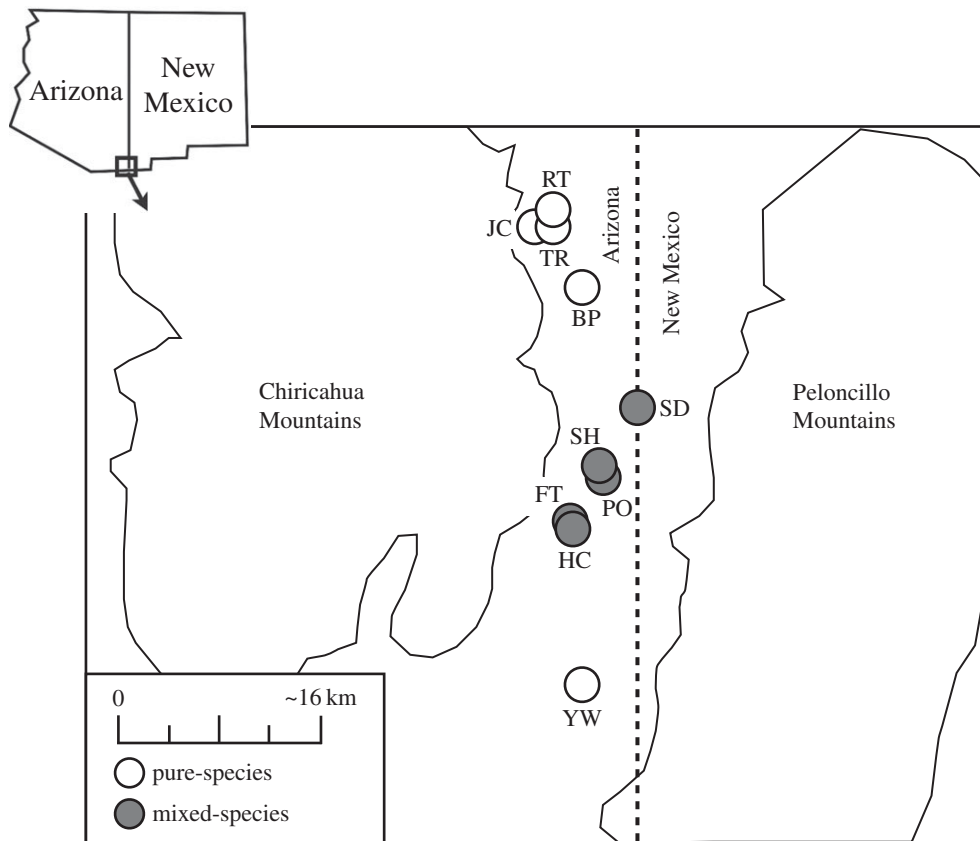


Figure 1. Schematic of study area in southwest USA showing the relative locations of populations where tadpoles were sampled for genetic analysis. Circles denoting populations are not to scale. Abbreviations correspond to population names in the electronic supplementary material, table S1, which provides the latitude and elevation measures used in the GESTE model. Females used in preference tests were collected at or near these same populations.

breeding or along roads near breeding sites in the area depicted in figure 1. Details of females used in the study are provided in the electronic supplementary material.

Females were returned to the University of North Carolina, where they were maintained on an ad libitum diet and a reverse light–dark cycle. All females were in the laboratory for at least eight months and were in reproductive condition (with eggs visible beneath the skin) prior to testing for their preferences. In two sets of experiments, we tested *S. multiplicata* females from pure- and mixed-species populations for their (i) ability to discriminate conspecific calls from heterospecific (*S. bombifrons*) calls and (ii) preferences for different conspecific male call rates. The former experiment evaluated whether *S. multiplicata* females in mixed- (as opposed to pure-) species populations are locally adapted to discriminate against *S. bombifrons*, as expected under the reinforcement hypothesis [5,6,45,46]. The latter experiment evaluated whether reinforcement acting in mixed-species populations has led to divergent preferences for a conspecific male trait in mixed- versus pure-species populations, as expected if reinforcement affects the expression of mate preferences for conspecific males [1,7–9,13,22,23,25,26]. That selection to discriminate *between* species concomitantly impacts female mate choice *within* species is a key mechanism by which reinforcement (and, more generally, reproductive character displacement) could promote divergence between conspecific populations in sympatry and allopatry [1,8,22].

We used phonotaxis tests as described previously [23,47] (for details, see the electronic supplementary material). To evaluate female ability to discriminate conspecifics from heterospecifics, we presented females with synthesized *S. multiplicata* versus synthesized *S. bombifrons* calls. The parameters of these stimuli consisted of species-typical values for each species [23]. Although a number of factors affect calling behaviour, including social interactions, temperature and introgression between *S. multiplicata* and *S. bombifrons* (K. Pfennig 1995–2013, unpublished data; see also

[23]), the calls of the two species are distinct, particularly in call rate [23] (see also supporting material in [47]). Our *S. multiplicata* stimulus consisted of calls at 34 calls min^{-1} , whereas the *S. bombifrons* stimulus consisted of calls at 73 calls min^{-1} , which is within the natural range of variation for these species in the San Simon Valley. In this experiment, a total of 69 females were tested (24 from at least three pure-species populations and 45 from at least six mixed-species populations).

In a separate experiment, we evaluated female choice for synthesized *S. multiplicata* calls that differed only in call rate. We focused on call rate because *S. multiplicata* females select mates using this call character, and female preferences for call rate differ between females from sympatric and allopatric regions [23]. Specifically, in a previous study [23], *S. multiplicata* females from sympatry preferred a slower call rate stimulus, whereas females from allopatry (outside of *S. bombifrons*'s range) preferred a faster call rate stimulus [23]. Based on these earlier results showing differences of mate preferences across different regions (i.e. collection sites separated by over 200 km), we therefore sought to determine whether preferences of females vary at the local scale of pure- versus mixed-species populations (figure 1). Thus, in two separate sets of tests, we presented the same females with choices between male calls at a rate of 30 versus 34 calls per minute and 34 versus 38 calls per minute. These stimulus sets spanned the same amount of variation (4 calls min^{-1}), and thereby presented females with a similar discrimination task. Moreover, the highest and lowest call rate stimuli approximated the 25th and 75th percentile for a combined distribution of male call recordings from both pure- and mixed-species populations. To date, we have no evidence that male calls differ between mixed- and pure-species populations (K. Pfennig 1995–2013, unpublished data).

All females were presented with both sets of stimuli, but the order in which they were presented was randomized. Moreover, for each female, at least 10 days elapsed between tests for the

alternative stimulus sets. We tested a total of 50 females (23 from at least three pure-species populations and 27 from at least five mixed-species populations).

(b) Statistical analysis of female mate preferences

We evaluated whether females preferred conspecific calls versus heterospecific calls within each population type using log-likelihood χ^2 -tests with a null 1:1 expectation. We then used contingency table analysis using a log-likelihood χ^2 -test to determine whether preferences for conspecifics versus heterospecifics differed between mixed- and pure-species populations.

We evaluated the prediction that females in the pure-species populations are more likely than females in mixed-species populations to prefer faster calls as follows. For the preferences of conspecific call rate, we report the number of females choosing each stimulus in the two different trials. However, because females were not independent across the trials, we compiled our data into three categories: females that preferred the faster call rate in *both* tests; females that preferred the faster call rate in *one* test; and females that preferred the faster call rate in *neither* test (i.e. these females preferred the slower stimulus in both tests). We then used contingency table analysis to determine whether the pattern of female preferences among these three groups differed between the pure- and mixed-population types.

Our *a priori* expectation was that females from pure-species populations would be more likely to prefer faster calls than would mixed-species females [23]. We therefore conducted a further analysis in which we compared preferences of only those females that exhibited a consistent preference across both tests (and thereby exhibited the strongest preferences for either the faster or slower call rate stimuli). For this analysis, we used a Fisher's exact test because the number of observations in some categories was less than 5.

(c) Gene flow within versus between pure- and mixed-species populations

If female preferences diverge between pure- and mixed-species populations, then females from either population type may be less likely to select males from the alternative population type as mates [8,11–13,17], thereby reducing gene flow.

We evaluated this possibility by estimating population structure between pure- and mixed-species populations. If gene flow between pure- and mixed-species populations is reduced relative to gene flow among populations *within* each type, then populations within each type should be more similar to each other in genotype frequencies than they are to the opposite population type [44]. However, this same pattern could also arise from the introgression between *S. multiplicata* and *S. bombifrons* in mixed-species populations [32]. We therefore controlled for introgression by identifying introgressed individuals and removing them from subsequent analyses.

We collected *S. multiplicata* and *S. bombifrons* tissues from five pure- and five mixed-species populations (figure 1), and from a pure *S. bombifrons* population within the region of sympatry (i.e. an allotopic population). We also collected tissues from one allopatric population per species (electronic supplementary material, table S1). We then genotyped approximately 20 individuals from each population (range 10–35 individuals, mean 19.4 ± 6.7 s.d.; electronic supplementary material, table S1) at 10 previously published microsatellite loci and one additional unpublished microsatellite locus (electronic supplementary material, table S2). See the electronic supplementary material for additional details.

To identify introgressed individuals in our samples from mixed-species populations, we used STRUCTURE v. 2.3.3 [48–50] to identify the most likely number of genetic clusters present in our data and to determine the most likely ancestry for each

individual. Because we included allopatric *S. multiplicata* and *S. bombifrons* as reference samples, we predicted that the number of genetic clusters should be two, with each species forming one cluster. Using the methods detailed in the electronic supplementary material, we found that, as predicted, the most likely number of genetic clusters was two (electronic supplementary material, table S3), and these clusters corresponded well with species identity (electronic supplementary material, figure S1). We therefore used a longer run of STRUCTURE to determine which individual samples from mixed-species populations were either pure *S. bombifrons* or individuals of mixed ancestry (see the electronic supplementary material). Any individual with a probability of membership in the *S. multiplicata* cluster of less than 0.75 was removed from subsequent analyses (electronic supplementary material, table S1).

Having controlled for introgression in the mixed-species populations, we tested for a reduction in gene flow between *S. multiplicata* populations that do versus do not co-occur with *S. bombifrons*. If gene flow is reduced between these population types, then mixed-species populations should be more similar in genotype frequencies to other mixed-species populations than they are to pure-species populations, and vice versa. We tested this prediction in two ways. First, we used an analysis of molecular variance (AMOVA) in ARLEQUIN v. 3.5.1.2 [51] to calculate hierarchical *F* statistics for the microsatellite genotypes. Individuals included in all ARLEQUIN analyses had a probability of belonging to the *S. multiplicata* genetic cluster greater than 0.75 (see above) and were missing data at no more than four loci (electronic supplementary material, table S1). We grouped the populations by type (i.e. pure- versus mixed-species; electronic supplementary material, table S1) and then calculated F_{SC} and F_{CT} for each locus and as a weighted average across loci. F_{SC} -values indicated the average level of gene flow occurring among populations within each population type. F_{CT} -values indicated any additional reduction in gene flow between the two population types, relative to levels among all the populations. To determine whether the southernmost, geographically isolated pure-species population (Yucca Wash, YW; electronic supplementary material, table S1; figure 1) was differentiated from the other pure-species populations, we used ARLEQUIN v. 3.5.1.2 [51] to calculate pairwise F_{ST} -values among all population pairs and estimated significance using 1000 permutations of the data. Finally, we performed a STRUCTURE analysis on this pure *S. multiplicata* dataset to assess whether pure- versus mixed-species populations tended to differ in their assignment to genetic clusters. See the electronic supplementary material for details of this analysis.

Based on results from MICRO-CHECKER [52] (see the electronic supplementary material), several population-locus combinations deviated from Hardy-Weinberg equilibrium, probably as the result of null alleles [53] (electronic supplementary material, table S2). Because null alleles could bias our results (if null alleles tended to be more prevalent in one population type versus the other), we repeated our AMOVA analysis after controlling for their effects. We used GENEPOP v. 4.2 [54] to estimate the frequency of any null allele at each locus in each population using maximum likelihood (EM algorithm [55]). We then re-ran locus-by-locus AMOVAs in ARLEQUIN using the corrected allele frequency data. We did not calculate global *F*-statistics with the corrected allele frequency data, because ARLEQUIN can only perform single-locus analyses on allele frequency data. Significance levels of the *F*-statistics were estimated using 50 000 permutations of the data.

Finally, we assessed the contributions of geographical location, population type and pond elevation for explaining population structure in the single-species *S. multiplicata* dataset. Populations within each type were geographically closer to other populations of the same type than to populations of the opposite type (figure 1). Populations of each type also were located at similar elevations (electronic supplementary material, table S1). Thus, geographical distance or elevation alone could potentially explain any

differences in allele and genotype frequencies found between the two population types. We used the program GESTE [56] to compare eight alternative models of population differentiation. GESTE uses a Bayesian method to estimate population-specific F_{ST} -values based on genetic data, and then relates these estimates to environmental explanatory variables using a generalized linear model framework [56]. In this analysis, we used allele count data that were corrected for the presence of null alleles using the Oosterhout correction algorithm implemented in MICRO-CHECKER [52]. The eight models we compared included different sets of explanatory variables: one model included only a constant, while the remaining models included the constant plus one, two or all three of the explanatory variables (i.e. population type, latitude, elevation). We used latitude as a proxy for geographical location because the populations are arrayed along a north–south axis (figure 1; electronic supplementary material, table S1). To estimate the model parameters, we ran GESTE for a total of 2 100 000 iterations, with a burnin of 100 000 iterations and a thinning interval of 20. If population type explains some of the population structure, then we would expect that the models including population type would have higher posterior probabilities than the models including only latitude and/or elevation.

3. Results

(a) Female mate preferences

We found that *S. multiplicata* females from mixed- and pure-species populations differed in their discrimination of conspecifics and heterospecifics. In mixed-species populations (where females risk hybridization with *S. bombifrons*), 30 out of 45 *S. multiplicata* females preferred conspecific calls over heterospecific calls, a pattern that was significantly different from a 1:1 random expectation (log-likelihood $\chi^2 = 5.10$, d.f. = 1, $p = 0.024$). By contrast, in nearby pure-species populations, 10 of 24 *S. multiplicata* females preferred conspecific calls over heterospecific calls, which was not different from a 1:1 random expectation (log-likelihood $\chi^2 = 0.670$, d.f. = 1, $p = 0.413$). These patterns of preference for conspecifics were significantly different between the two population types (log-likelihood $\chi^2 = 4.01$, d.f. = 1, $p = 0.045$). Thus, in mixed-species populations, reinforcement has contributed to locally adapted *S. multiplicata* mate preferences that minimize hybridization with *S. bombifrons*.

When *S. multiplicata* females were presented with 30 versus 34 calls per minute, 16 of 23 females from the pure-species populations preferred the faster call rate, whereas nine of 24 females from the mixed-species populations preferred the faster call rate. When females were presented with call rates of 34 versus 38 calls per minute, 15 of 21 females from the pure-species populations preferred the faster call rate, whereas 11 of 24 females from the mixed-species populations preferred the faster call rate. Combining these results across both tests, we found that, in the pure-species populations, 11 females preferred the faster call rate in both tests, seven preferred the faster call rate stimulus in one test and three preferred the slower call rate stimulus in both tests. In the mixed-species populations, we found that three females preferred the faster call rate in both tests, 11 preferred the faster call rate stimulus in one test and seven preferred the slower call rate in both tests. These patterns of preference were significantly different between the population types (log-likelihood $\chi^2 = 7.40$; d.f. = 2; $p = 0.025$).

When we contrasted only those females above that consistently chose the faster or slower call rate in both tests (i.e. those

females that exhibited the strongest preferences for call rate), we found that females from pure- and mixed-species populations significantly differed in their preferences for call rate (Fisher's exact two-sided $p = 0.035$). These results indicate that, as expected from previous work [23], females from pure-species populations have a greater propensity to prefer faster call rates than females from mixed-species populations.

(b) Gene flow within versus between pure- and mixed-species populations

We next asked whether gene flow was significantly reduced between these two population types. The STRUCTURE analysis identified a total of 26 individuals with a probability of membership in the *S. multiplicata* genetic cluster of less than 0.75 (electronic supplementary material, table S1 and figure S1). Most of these ($n = 17$) had a probability of membership in the *S. multiplicata* genetic cluster of less than 0.05, indicating that they were likely to be pure *S. bombifrons* tadpoles. All 26 of these samples were removed from further analyses (electronic supplementary material, table S1).

The AMOVA indicated that gene flow between pure- and mixed-species populations was reduced relative to levels of gene flow among populations within the same type. Because three loci (SpeaD103, SpeaD7 and Sm1) did not amplify in any individuals from one population (Four Ten; electronic supplementary material, table S1), the global F -statistics were calculated as a weighted average across all 10 populations for the remaining eight loci. For these eight loci combined, we found significant population structure within each population type ($F_{SC} = 0.073$, $p < 0.00001$), which suggests that gene flow is significantly reduced among populations within each type relative to the null expectation of panmixia. More critically, we found that gene flow is reduced between pure- and mixed-species populations: grouping populations by type explained a significant amount of variation in genotype frequency ($F_{CT} = 0.009$, $p = 0.047$). Indeed, although one of our pure-species populations (YW; electronic supplementary material, table S1; figure 1) is geographically isolated from the remaining pure-species populations (with the mixed-species populations in the intervening area; figure 1), pairwise F_{ST} -values indicate this population was significantly differentiated from three of the mixed-species populations (electronic supplementary material, table S4). However, this same population was not significantly differentiated from any of the more distant pure-species populations (electronic supplementary material, table S4). Thus, populations of each type tend to be more similar in genotype frequencies than expected by chance, which is consistent with reduced gene flow between pure- and mixed-species populations.

At the individual loci, nine of 11 loci exhibited significant population structure within each population type without correcting for null alleles (uncorrected F_{SC} , table 1), and all 11 loci exhibited significant population structure within each population type after correcting for null alleles (corrected F_{SC} , table 1). One locus (Sm14) also exhibited a significant signature of reduced gene flow between pure- and mixed-species populations (uncorrected F_{CT} , table 1), whereas a second locus (SpeaD111) showed a marginally non-significant F_{CT} . For Sm14, this signature became non-significant at the $\alpha = 0.05$ level after correcting for null alleles. Correcting for null alleles had little effect on SpeaD111 (table 1). Note that uncorrected and corrected F_{SC} and F_{CT} values for loci SpeaD103, SpeaD7 and Sm1

Table 1. Uncorrected and corrected F_{SC} and F_{CT} -values for individual loci and as global averages over all sampled populations.

	Sb8	SpeaC7	SpeaD111	Sm4	Sb15	Sm25	SpeaD103 ^a	SpeaD7 ^a	Sb28	Sm14	Sm1 ^a	global (weighted average over eight loci, all 10 populations)
uncorrected F_{SC}	0.195 (0.000)	0.077 (0.001)	0.077 (0.000)	0.084 (0.000)	0.077 (0.000)	0.055 (0.000)	0.042 (0.039)	0.051 (0.073)	0.052 (0.018)	0.021 (0.279)	0.075 (0.004)	0.073 (0.000)
(<i>p</i> -value)												
corrected F_{SC}	0.175 (0.000)	0.045 (0.000)	0.068 (0.000)	0.058 (0.000)	0.060 (0.000)	0.057 (0.000)	0.054 (0.002)	0.159 (0.000)	0.090 (0.000)	0.045 (0.000)	0.107 (0.000)	
(<i>p</i> -value)												
uncorrected F_{CT}	-0.036 (0.683)	0.004 (0.273)	0.022 (0.087)	0.011 (0.201)	0.001 (0.261)	-0.001 (0.359)	-0.021 (1.000)	0.022 (0.204)	-0.001 (0.372)	0.041 (0.014)	0.001 (0.489)	0.009 (0.047)
(<i>p</i> -value)												
corrected F_{CT}	-0.039 (0.816)	0.001 (0.307)	0.021 (0.078)	0.005 (0.213)	-0.001 (0.344)	-0.003 (0.403)	-0.009 (0.571)	0.042 (0.366)	-0.019 (0.795)	0.020 (0.072)	0.048 (0.127)	
(<i>p</i> -value)												

^aLoci for which one mixed-species population had completely missing data, so F -values were calculated with only nine populations. The global average excludes these loci.

Table 2. Posterior probabilities for alternative GESTE models of population differentiation.

model	explanatory variables included	posterior probability
1	constant	0.597
2	constant and population type	0.173
3	constant and latitude	0.047
4	constant, latitude and population type	0.017
5	constant and elevation	0.070
6	constant, latitude and elevation	0.001
7	constant, population type and elevation	0.081
8	constant, latitude, population type and elevation	0.007

are based on genotypes at four mixed-species populations and five pure-species populations because these loci failed to amplify in one mixed-species population, as noted above.

The GESTE model with the highest posterior probability included only the constant (table 2), indicating that none of the three explanatory variables was strongly related to the observed population structure. However, among the models that included explanatory variables, the model that included the effect of population type only was approximately twice as probable as the next most likely model (table 2). Thus, whether a population contains heterospecifics *per se* is a better explanation of population structure than other variables associated with elevation or geographical location.

4. Discussion

We evaluated whether neighbouring populations that differ in the presence of heterospecifics have evolved divergent mate preferences owing to reinforcement acting in populations where heterospecifics are encountered. We also evaluated whether gene flow is significantly reduced between these population types, as is expected if divergent mating behaviours contribute to reproductive isolation.

We found that *S. multiplicata* females from nearby populations that do and do not occur with *S. bombifrons* showed divergent mate preferences. In particular, females from mixed-species populations (where hybridization with *S. bombifrons* can occur) discriminated conspecifics from heterospecifics, but females from pure-species populations did not. This pattern is a hallmark of reinforcement [5,45,46], and is consistent with previous evidence indicating that *S. multiplicata* has undergone reinforcement of mate preferences in mixed-species populations [23,24,36]. We also found that females from mixed- and pure-species populations express divergent preferences for conspecific male call characters. These divergent preferences are likely to be the result of reinforcement, because the evolution of enhanced discrimination of conspecifics from heterospecifics will often concomitantly affect patterns of mate choice among conspecifics [1,8,10,22,57]. That mixed- and pure-species populations exhibit such divergent preferences is significant, because it indicates that female preferences can become locally adapted to the presence of heterospecifics, even at a relatively fine spatial scale.

Our finding that female preferences are divergent between pure- and mixed-species populations suggests that male calls should also diverge at this local scale. However, introgression with *S. bombifrons* in the mixed-species ponds [31,32] appears to increase the call rates in *S. multiplicata* males of mixed ancestry, and thereby obscure differences in the distributions of calls between population types (K. Pfennig 2002–2013, unpublished data). Evaluating the relative impacts of female preferences, gene flow between species and other factors (such as temperature and social interactions) that can affect male call traits requires further study.

We further found that conspecific populations of *S. multiplicata* from pure- and mixed-species populations showed a significant reduction in gene flow relative to that within each population type. Moreover, population type explains more of the observed population structure than does geographical distance or elevation differences between populations. Although significant, the magnitude of the reduction in gene flow between population types was not large. This is unsurprising for at least two reasons. First, we found significant differentiation among individual populations within each population type at nearly every locus (table 1), and any additional reduction between populations of the opposite type is likely to be small. Indeed, the fact that the GESTE model with the constant alone was the most probable model (table 2) is consistent with individual population differentiation explaining most of the structure. Second, because divergence with gene flow produces heterogeneous genomic divergence [58], reduced gene flow—even when it exists—may not always be detectable using randomly identified genetic markers (such as microsatellites), which are potentially neutral [59]. Future work is needed to determine whether signatures of reduced gene flow are even stronger at markers linked to traits (such as mating behaviour) that experience divergent selection between pure- and mixed-species populations.

The observed pattern of reduced gene flow is expected if divergent preferences contribute to reproductive isolation between pure- and mixed-species populations. Yet this pattern could also be produced by *ecological* character displacement contributing to reproductive isolation between pure- and mixed-species populations [43,44]. In particular, the tadpoles of *S. multiplicata* and *S. bombifrons* compete for resources, and previous work has shown that tadpoles in pure- and mixed-species populations have diverged in resource-use traits [41,43,60–62]. Indeed, this divergence results in reduced fitness of offspring from crosses between individuals from pure- and mixed-species populations [43], which may have contributed to reduced gene flow between these populations [43]. However, although ecological character displacement may explain some of the observed reproductive isolation between *S. multiplicata* in pure- versus mixed-species populations, our preference data suggest that reproductive character displacement (specifically, reinforcement) also contributes to divergence in mating behaviours that could serve as a pre-mating reproductive isolating barrier between these population types. Additional work is needed to evaluate the relative contributions of these two forms of character displacement in promoting divergence and reproductive isolation in this and similar systems [1,2]. Generally, species that are similar enough to compete for resources are also likely to interact reproductively. Consequently, both forms of character displacement are likely to occur together and simultaneously promote divergence between sympatry and allopatry [1,2].

Low gene flow and selective trade-offs between sympatric and allopatric populations are complementary factors that enhance the likelihood that reinforcement would drive conspecific populations in sympatry versus allopatry to diverge and become reproductively isolated [1,14]. In spadefoots, both factors potentially contribute to the observed patterns. We found that, despite their proximity, populations of *S. multiplicata* display significant structure within population type (see Results; tables 1 and 2; see also [43]), indicating that gene flow is low among them. Such low gene flow could stem from selection against migrants among the different populations (especially those of different types [16,63]). Regardless of its cause, when gene flow is low, reinforcement can more readily promote reproductive isolation of conspecific populations that differ in interactions with heterospecifics [14,18,20].

In addition to reduced gene flow, selective trade-offs between sympatric and allopatric populations probably contribute to divergence between pure- and mixed-species populations. Previous work has shown that, by avoiding heterospecific males, sympatric *S. multiplicata* females have evolved mate preferences that preclude them from choosing high-quality conspecific mates [21,23,24]. Such preferences would be disfavoured in allopatry, where females use fast call rates as indicators of male quality and condition [23]. Likewise, allopatric preferences would be disfavoured in sympatry because they place females at greater risk of hybridization [23]. Thus, existing population structure, coupled with selective trade-offs favouring divergent preferences in sympatry and allopatry, have probably contributed to the significantly reduced gene flow between pure- and mixed-species populations described here.

To date, the focus on reinforcement has been on its role in finalizing the speciation process. When interactions with heterospecifics are costly (as when hybridization results in offspring of low fitness), selection should favour behaviours that prevent reproductive interactions, thereby enhancing isolation between species (or incipient species) to the point where gene exchange between them is reduced or completely eliminated [3,36,38,39]. Yet reinforcement (and, more generally, reproductive character displacement) may also serve to *initiate* divergence—and possibly speciation [1,2,6–17,43]. Indeed, if reinforcement occurs with different heterospecifics in different parts of a focal species's range, or in different ways across sympatry and allopatry, it can contribute to 'speciation cascades' in which multiple speciation events are triggered by reinforcement [1,8,13], a process that has also been called the 'cascade reinforcement hypothesis' and 'RCD speciation' [9,15,16,20] (see also [6]). That reinforcement can initiate speciation remains controversial [18,19], despite empirical and theoretical studies suggesting that it can contribute to population divergence and reproductive isolation [8,10–13,17]. Our work highlights the possibility that reinforcement can not only initiate reproductive isolation between conspecific populations, but also that it can do so even among neighbouring populations that differ in exposure to heterospecifics with which mating is costly. Thus, rather than solely contributing to the final phase of speciation, reinforcement and, more generally, reproductive character displacement may play a crucial role in initiating the speciation process.

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