

## A TEST OF ALTERNATIVE HYPOTHESES FOR THE EVOLUTION OF REPRODUCTIVE ISOLATION BETWEEN SPADEFOOT TOADS: SUPPORT FOR THE REINFORCEMENT HYPOTHESIS

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**Abstract.**—How do species that interbreed become reproductively isolated? If hybrids are less fit than parental types, natural selection should promote reproductive isolation by favoring the evolution of premating mechanisms that prevent hybridization (a process termed reinforcement). Although reinforcement should generate a decline in hybridization over time, countervailing forces of gene flow and recombination are thought to preclude natural selection from enhancing and finalizing reproductive isolation. Here, I present recent estimates of hybridization frequency between two species of spadefoot toad, *Spea multiplicata* and *S. bombifrons*. I compare these recent measures of hybrid frequency with previously published estimates and show that hybridization between these species has declined precipitously over the past 27 years. Although previous studies suggest that reinforcement possibly accounts for this decline in hybrids over time, three alternative hypotheses also can explain the observed decrease in hybridization. First, if one of the two interacting species becomes rare, opportunities for and incidence of hybridization may decrease. Second, if one of the two interacting species is initially rare, hybridization may be initially common if the rare species has difficulty locating conspecific mates. Third, if hybrids are produced only in particular environments, hybrid frequency may decline if habitat changes result in loss of those environments that promote hybrid formation. I found no support for these three alternative explanations of the decline in hybrids. Instead, reinforcement appears to best account for the evolution of enhanced reproductive isolation between these species. Moreover, the finding that hybridization declined precipitously in only 27 years suggests that many systems that have undergone reinforcement may be overlooked because reproductive isolation between the interacting populations or species may already be complete.

**Key words.**—Environmental variation, hybridization, mate choice, reinforcement, *Spea*, speciation.

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The evolution of reproductive isolation between potentially interbreeding species or recently diverged populations is a necessary component of both the speciation process and the maintenance of species barriers. Without reproductive isolation, interbreeding would tend to break down evolved differences between species or recently diverged populations. Evaluating how reproductive isolating mechanisms evolve is therefore critical to understanding how new species form and how barriers between species are maintained.

One mechanism by which reproductive isolating barriers may evolve is via natural selection. If hybrids are unfit relative to pure-species types, natural selection should favor the evolution of premating isolating mechanisms—usually mating behaviors—that prevent the production of unfit hybrids (e.g., Coyne and Orr 1989; Howard 1993; Noor 1995; Saetre et al. 1997). This process, termed reinforcement, should result in a decline of hybridization over time if the evolution of premating isolating barriers effectively enhances reproductive isolation between the two interbreeding populations or species (Dobzhansky 1940; Jones 1973; Blair 1974; Britch et al. 2001).

Reinforcement should finalize speciation by generating isolating mechanisms that prevent hybridization between species or recently diverged populations (Dobzhansky 1940). Yet, many have argued that natural selection is incapable of promoting and finalizing reproductive isolation between species or divergent populations via the reinforcement process (reviewed in Howard 1993; Butlin 1995). Instead, gene flow

and recombination are expected to counteract the effects of natural selection, thereby precluding the evolution of enhanced reproductive isolation (Barton and Hewitt 1989; Kelly and Noor 1996; Servedio and Kirkpatrick 1997; Kirkpatrick and Servedio 1999; Barton 2001; Britch et al. 2001). Consequently, hybrid zones are expected to be stable and persistent over time. Such stability results from the counterbalancing effects of natural selection, which promotes reproductive isolation, and gene flow, which promotes the breakdown of reproductive isolation (Barton and Hewitt 1989).

Finding that hybrid frequency declines over time when there is selection against hybrids would provide strong support for the hypothesis that natural selection can promote reproductive isolation between species. However, this critical prediction that hybrids should decline over time in response to selection against hybrids remains largely untested (but see Jones 1973; Blair 1974; Britch et al. 2001) or unsupported (Britch et al. 2001).

Moreover, although a decline in hybridization is consistent with the reinforcement hypothesis, hybridization may decline over time for a variety of reasons other than reinforcement. Hybridization may decline when frequency of encounters with either conspecifics or heterospecifics changes over time (for a review see Wirtz 1999; for an example of hybridization frequency related to abundance of conspecifics see Malmos et al. 2001). Hybridization may also decline when habitats that promote hybrid formation change (for examples where hybrids are favored in particular habitats see Arnold 1997; Neuffer et al. 1999; Orians et al. 1999; Parris 2001; Pfennig and Simovich 2002). Similarly, hybridization may decline when reproductive timing or behaviors change for reasons other than avoidance of hybridization (for examples of hy-

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bridization precluded by different timing of reproduction see Knowlton et al. 1997; Monti et al. 1997; Soliva and Widmer 1999; Simon et al. 2000; Miyatake 2002; Thomas et al. 2003). Additionally, hybridization may decline when one or both species shifts hosts or habitat use for reasons other than avoidance of hybridization (e.g., Thomas et al. 2003; see also reviews and references therein by Feder 1998; Menken and Roessingh 1998). Finally, competition for resources between interacting species may promote specialization on alternative resources that concomitantly minimizes encounters between the two species and thereby reduces the frequency of hybridization between them (reviewed in Schluter 2000).

Here, I evaluate several hypotheses other than reinforcement that can explain decreases in hybrid frequency over time. In particular, I focus on the three hypotheses that most likely apply to the study system with which I worked.

First, if one of the two interacting species becomes rare, opportunities for and incidence of hybridization may decrease. This hypothesis is essentially a statistical argument, which posits that hybrid frequency is related to the probability of encountering heterospecifics: if heterospecifics are common, hybrids will be common, whereas if heterospecifics are rare, hybrids will be rare.

A second hypothesis for a decline in hybrids over time is that if one of the two interacting species is initially rare, hybridization may be initially common if the rare species has difficulty locating conspecific mates (reviewed in Wirtz 1999; e.g., Malmos et al. 2001). In contrast to the first hypothesis, this second hypothesis suggests that changes in behavior may mediate hybridization frequency. If conspecific mates are rare or unavailable, an individual may opt to mate with a heterospecific rather than forego mating all together. When conspecifics are common, however, individuals are expected to prefer conspecific versus heterospecific mates. According to this hypothesis, the frequency of hybridization should be inversely related to the frequency of conspecific mates available.

Finally, if hybrids are produced only in particular environments, hybrid frequency may decline if habitat changes result in loss of those environments that promote hybrid formation. Evaluating whether reinforcement or any of these alternative mechanisms contribute to declines in hybrid frequency over time is necessary for understanding how reproductive isolation may evolve.

I used spadefoot toads (*Spea multiplicata* and *S. bombifrons*) as a system for evaluating whether hybrid frequency has changed over time. Previous studies have revealed that these species appear to be undergoing reinforcement, so my a priori prediction was that hybridization between the two species should have declined over time. To evaluate this prediction, I compared recent estimates of  $F_1$  hybrid frequency with previously published measures of  $F_1$  hybrid frequency from the past 27 years. Because I found that hybrids declined over time (see Results), I evaluated whether each of the three alternative hypotheses discussed above could account for the observed decline in hybridization. I focused on these hypotheses because they are the most likely alternatives to reinforcement that can account for the decline in hybrids over time in this study system. By evaluating these hypotheses, I

was able to determine whether the decline in hybrids could be attributed to a mechanism other than reinforcement.

## MATERIALS AND METHODS

### *Study System*

In southeastern Arizona, two species of spadefoot toad, *S. multiplicata* and *S. bombifrons*, inhabit the same areas and historically have hybridized. These two species are the most divergent of those in the genus *Spea* (Wiens and Titus 1991). The adults of these species risk hybridization when they congregate in ephemeral ponds to breed. Breeding is highly explosive, with both species breeding simultaneously in the same ponds for only one to a few nights per year following summer rainstorms (Bragg 1965; K. Pfennig, pers. obs.).

Prior work revealed two pieces of evidence that natural selection may be favoring the evolution of enhanced reproductive isolation (i.e., reinforcement) between these species. First, hybrid offspring tend to be less fit than pure-species types (Simovich 1985; Simovich et al. 1991; Pfennig and Simovich 2002), thereby generating natural selection against hybrid genotypes. Specifically,  $F_1$  hybrid males are sterile and  $F_1$  hybrid females are less fecund than pure-species females (Simovich 1985; Simovich et al. 1991). Additionally, some  $F_1$  hybrid offspring have lower survival and slower development than pure-species offspring (Pfennig and Simovich 2002). Second, perhaps as a consequence of the reduced fitness of hybrid types, mating behaviors that inhibit matings with heterospecifics exist in sympatry. In contrast to allopatric *S. multiplicata* females, *S. multiplicata* females in sympatry with *S. bombifrons* select against males that possess mating calls resembling those of *S. bombifrons* males (Pfennig 2000). Moreover, the mating calls of both *S. multiplicata* males and *S. bombifrons* males are more dissimilar from those of the other species where the two species coexist than where the two species do not coexist (Pierce 1976; K. Pfennig, unpubl. data).

If selection generally favors the evolution of mating behaviors that prevent the production of hybrids, then the frequency of hybrids between *S. multiplicata* and *S. bombifrons* is predicted to decline over time. As mentioned above, however, three mechanisms other than reinforcement could also explain changes in hybridization frequency between *S. multiplicata* and *S. bombifrons*.

First, the rarer of the two species, *S. bombifrons*, may have decreased in frequency over time, thereby reducing the opportunities for hybridization. As described above, this statistical argument predicts an inverse correlation between the frequency of *S. bombifrons* and time.

A second factor that may contribute to changes in hybridization frequency between *S. bombifrons* and *S. multiplicata* is that *S. bombifrons* may hybridize when conspecific mates are rare or unavailable but mate preferentially with conspecifics as conspecific mates become more abundant. Thus, *S. bombifrons* are expected to modify their behavior depending on the availability of conspecific mates at a given breeding aggregation. Consequently, this hypothesis critically predicts that the frequency of hybridization at a given breeding event will be inversely correlated with the frequency of breeding *S. bombifrons*.

A final factor that may contribute to a change in hybrid frequency between *S. bombifrons* and *S. multiplicata* is that abiotic conditions contributing to hybrid formation may have changed over time. Although *S. multiplicata* females are always under selection to avoid hybridization, *S. bombifrons* females may sometimes benefit from hybridizing with *S. multiplicata* during times of lower rainfall (i.e., when breeding ponds are most ephemeral; Simovich 1985; Simovich et al. 1991; Pfennig and Simovich 2002). In particular, hybrid tadpoles of *S. bombifrons* females develop faster and are more likely to escape a drying pond than pure *S. bombifrons* tadpoles (Simovich 1985; Simovich et al. 1991; Pfennig and Simovich 2002). In partial support of this hypothesis, *S. bombifrons* females are more likely than *S. multiplicata* females (or *S. bombifrons* males) to hybridize (Simovich 1985; Pfennig and Simovich 2002). Moreover, hybrid offspring are more frequent in more ephemeral pools (Simovich 1985; Pfennig and Simovich 2002). If rainfall has increased over time, then *S. bombifrons* females may not encounter the ephemeral habitats in which hybrid offspring outperform parental types. Consequently, *S. bombifrons* females may be less likely to hybridize in increasingly wetter habitat.

Although resource competition can result in reduced hybridization if two potentially interbreeding species specialize on alternative resources and therefore come into contact only rarely, this explanation for reduced hybridization does not appear to apply to the spadefoots. Although the tadpoles of *S. multiplicata* and *S. bombifrons* reveal ecological character displacement in their feeding morphology as a consequence of resource competition (Pfennig and Murphy 2000, 2002, 2003), the adults of both species breed in the same ponds and risk hybridization. Thus, specialization on alternative resources does not likely explain reduced hybridization in this system.

#### Recent Estimates of Hybrid Frequency in the Field

I estimated hybrid frequency between *S. multiplicata* and *S. bombifrons* during the summer months of June, July, and August from 1999 to 2001 to obtain recent estimates of hybridization between the two species. To do so, I collected tadpoles from seven breeding aggregations in which both *S. multiplicata* and *S. bombifrons* were known to have occurred near Portal, Arizona. The sites of these aggregations are presented in Figure 1. Sampling took place approximately five to eight days following a breeding event (spadefoots breed on a single night). The number of tadpoles collected from each pond ranged from 17 to 48. Tadpoles were collected from throughout each pond with hand-held dip nets, so that a representative sample of the tadpoles in each pond was obtained. Following collection, the tadpoles were killed by immersion in a 0.1% aqueous solution of tricane methane-sulfonate (MS 222) and stored in individually labeled sample bags at  $-80^{\circ}\text{C}$ .

The tadpoles were identified as  $F_1$  hybrids or pure-species types using standard protocols for cellulose acetate protein electrophoresis (Hebert and Beaton 1993). Four markers were used to characterize each tadpole as a pure-species or hybrid type: isocitrate dehydrogenase (Idh)-1 and -2, lactate dehydrogenase (Ldh)-1, and malate dehydrogenase (Mdh)-1.

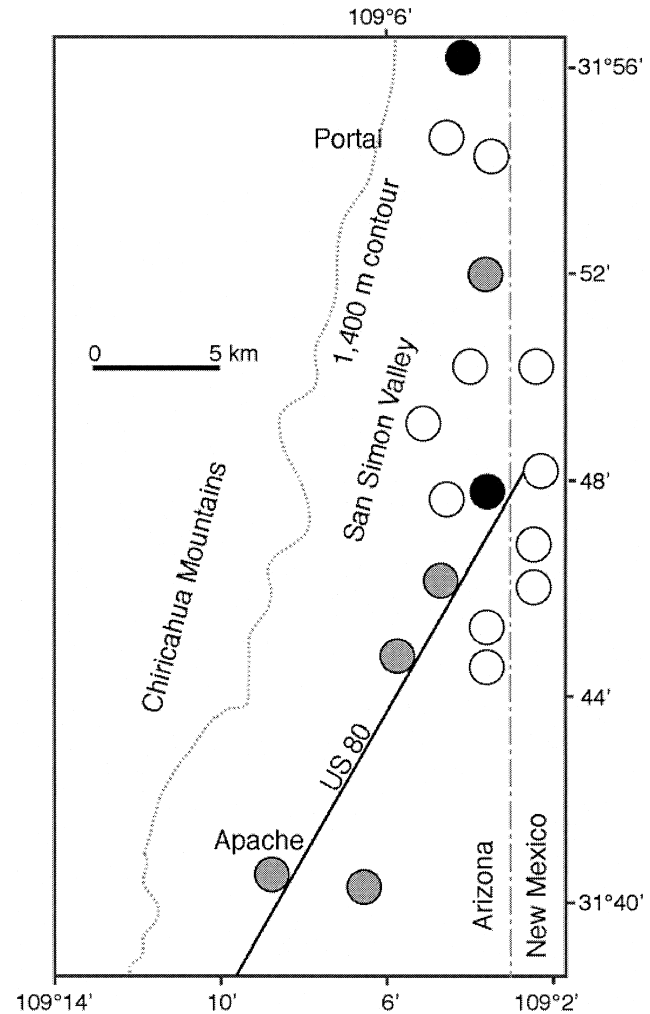


FIG. 1. Collection localities of tadpoles used to estimate hybrid frequency. Gray circles show ponds sampled during 1999–2001. White circles show ponds sampled by Simovich (1985) during 1980–1982. Black circles show locations sampled in both studies. Some ponds were sampled in multiple years.

These loci segregate independently and are divergent between *S. multiplicata* and *S. bombifrons* (Sattler 1985; Simovich 1985; Simovich and Sassaman 1986). These markers can also be used to identify complex hybrids beyond the  $F_1$  (Simovich and Sassaman 1986), but no backcross offspring were found in any of the samples (see Results). I therefore focus only on estimates of  $F_1$  hybrid frequency in comparing hybrid frequency across different time periods (see below). The frequency of  $F_1$  hybrids measures how frequently hybridization occurs between the two species, which, according to the reinforcement hypothesis, should decline over time.

I generated relative frequencies of each species and  $F_1$  hybrids for each pond sampled. By sampling tadpoles produced from different breeding events, I could measure the frequency of  $F_1$  hybrids and pure-species types that were actually produced in a given year. Because breeding events are discrete, they serve as independent opportunities for hybridization to occur. Thus, the relative measures of hybrids

TABLE 1. Mean ( $\pm$  SEM) proportion of F<sub>1</sub> hybrids (*Spea multiplicata*  $\times$  *S. bombifrons*) and pure-species types for the time periods in which populations near Portal, Arizona, were sampled. Note that the estimates prior to 1999 do not add to one because backcrosses that were observed in previous years are not included in the table. *N*, the number of independent estimates of F<sub>1</sub> hybrid and pure-species frequencies derived from each study. These independent estimates are used as replicates for all analyses. Citations from which data prior to 1999 are drawn are provided in Materials and Methods.

Time period	<i>N</i>	Proportion F <sub>1</sub> hybrids	Proportion <i>S. bombifrons</i>	Proportion <i>S. multiplicata</i>
1975–1977	1	0.057	0.057	0.857
1980–1982	26	0.049 (0.016)	0.154 (0.031)	0.767 (0.037)
1990–1992	3	0.019 (0.011)	0.352 (0.124)	0.609 (0.118)
1999–2001	7	0.003 (0.003)	0.339 (0.078)	0.658 (0.076)

and pure-species types from the different ponds provide replicate measures of hybridization in a given year.

#### Comparison of Recent Estimates of Hybrid Frequency with Earlier Studies

To determine whether hybrid frequency between *S. multiplicata* and *S. bombifrons* has changed over time, I compared the estimates of F<sub>1</sub> hybrid frequency obtained above with the best published estimates of hybrid frequency from the same populations that I sampled near Portal, Arizona. In particular, I obtained F<sub>1</sub> hybrid frequency estimates from three earlier studies. All but one of these studies limited population sampling to the area near Portal; for the one study that sampled populations elsewhere, I used only the data pertaining to populations near Portal. I focused on these populations because these are the populations for which both the mating behavior of *S. multiplicata* and *S. bombifrons* and the fitness consequences of hybridization between the two species are known (Simovich 1985; Simovich et al. 1991; Pfennig 2000; Pfennig et al. 2000; Pfennig and Simovich 2002).

The published estimates of F<sub>1</sub> hybrid frequency consisted of two studies that estimated hybrid frequency based on tissues collected from adults and one study that obtained estimates from collections of tadpoles produced from independent breeding events. I describe these studies and the type of data I obtained from each below. Details of sampling protocols and genetic analyses used in identifying hybrids are described in each account. In all studies, hybrids were identified using the same allozyme loci (Idh-1 and -2, Ldh-1, and Mdh-1) described above. Although each of the studies described below provided frequency estimates of backcrosses in addition to F<sub>1</sub> hybrids and pure-species types, I used only the estimates of F<sub>1</sub> hybrid frequency and pure-species types.

The earliest study using genetic markers to estimate hybrid frequency between *S. multiplicata* and *S. bombifrons* is that of Sattler (1985). This study provided one pooled estimate of the relative frequencies of F<sub>1</sub> hybrids and pure-species adults for the entire period of 1975–1977 (Table 1). A second study (Crosswaite 1994) also sampled adults, but provided estimates of F<sub>1</sub> hybrid and pure-species frequencies for each year sampled from 1990–1992. I therefore obtained three estimates of F<sub>1</sub> hybrid and pure-species frequencies for the period of 1990–1992 (Table 1). This latter study also pro-

vided estimates of hybrid and pure-species frequencies broken down by the particular sites where adults were collected. I used the reported yearly pooled estimates from this study, however, because so few adults were sampled in some sites (as few as two animals in some sites) that per site measures of hybrid frequencies could be highly biased. Because some F<sub>1</sub> hybrid offspring may have lower survival than pure-species offspring (Pfennig and Simovich 2002), samples of adults potentially underestimate the frequency of F<sub>1</sub> hybrids produced. Thus, estimates of F<sub>1</sub> hybrids in these time periods are potentially conservative. Concomitantly, the relative frequency of pure-species offspring actually produced may be overestimated by adult sampling.

The remaining study (Simovich 1985) used samples of tadpoles to assess hybrid and pure-species frequencies of *S. multiplicata* and *S. bombifrons*. In all cases I only used estimates of hybrid frequency from ponds in which both species were known to have occurred. I obtained from Simovich (1985) the relative frequencies of hybrid and pure-species tadpoles from each of 26 breeding events for the time period 1980–1982 (see also Pfennig and Simovich 2002). As I described above, such estimates provide independent replicate measures of the frequency of hybrids and parental types actually produced at several breeding aggregations within a given year.

The number of tadpoles collected by Simovich (1985) from throughout each of these ponds ranged from 23 to 737. Although Simovich (1985) collected on average 185 tadpoles from these ponds, which was greater than the number of tadpoles collected per pond in 1999–2001, the samples in both time periods were representative of the ponds sampled. Of the seven ponds sampled in 1999–2001, two were the same as those breeding aggregations sampled by Simovich (1985; Fig. 1). The remaining five ponds were in close proximity (within 10 km) to the ponds sampled in 1980–1982.

To evaluate if hybrid frequency had declined with time, I combined my own estimates of hybrid frequency with those from the literature described above. Because the data did not meet parametric assumptions, I used a Spearman nonparametric rank order correlation analysis to determine if the estimates of F<sub>1</sub> hybrid frequency were inversely associated with time period during which hybrids were sampled. This analysis is based on ranks of the data, so it is not sensitive to outliers present in the data (Zar 1999).

I next generated mean estimates of hybrid frequency. For the 1980–1982 and 1999–2001 time periods in which tadpoles at independent ponds were sampled, I calculated the mean relative frequency of pure-species and hybrid tadpoles using the relative measures of these types from each pond as replicates. For 1990–1992, during which adults were sampled, I calculated the mean relative frequency of pure-species and hybrid adults by averaging the three yearly estimates of relative pure-species and hybrid frequency (see above). Finally, because only one pooled estimate of the relative frequencies of hybrid and pure-species types was provided for 1975–1977, I use this estimate in all subsequent analyses of the means.

To explore how mean frequencies of hybrids compared across the different time periods, I used a nonparametric Kruskal-Wallis analysis of variance corrected for tied ranks

(Zar 1999). I next used Dunn's nonparametric procedure for comparisons of a control to other groups (Zar 1999) to compare the mean estimate of hybrid frequency during 1999–2001 with the mean estimates of the two previous time periods (1980–1982 and 1990–1992). Because I had only one estimate of hybrid frequency for 1975–1977, I did not compare this time period with my mean estimate of hybrid frequency during 1999–2001.

#### *Evaluation of Alternative Hypotheses for Hybrid Decline*

Because I found a significant decline in hybrid frequency over time (see Results), I evaluated whether the three mechanisms other than reinforcement that were described above can account for the observed decrease in hybrid frequency between *S. multiplicata* and *S. bombifrons*.

#### *Alternative 1: opportunities for hybridization have declined*

If the observed decline in  $F_1$  hybrids resulted from decreased opportunities for hybridization, I expected the less abundant species, *S. bombifrons*, to have become rarer over time. To evaluate this possibility, I used a Spearman nonparametric rank order correlation analysis to determine if the estimates of *S. bombifrons* frequency were inversely associated with time period. I used this nonparametric test because the data did not meet parametric assumptions. To further explore how the mean frequencies of *S. bombifrons* compared across the different time periods, I used the same statistical methods that I used to compare mean estimates of  $F_1$  hybrid frequency over time (see above).

#### *Alternative 2: hybridization is initially common when one species is rare*

A second hypothesis suggests that the rarer species, *S. bombifrons*, may hybridize when conspecific mates are rare or unavailable but mate preferentially with conspecifics when conspecific mates are available. This hypothesis posits that *S. bombifrons* modify their behavior depending on the availability of conspecific mates at a given breeding aggregation. Thus, this hypothesis critically predicts that the frequency of hybrid offspring produced in a given breeding aggregation should be inversely correlated with the frequency of breeding *S. bombifrons* adults attending that aggregation.

To test this prediction, I assessed whether  $F_1$  hybrid production was associated with the frequency of breeding *S. bombifrons* adults. To do so, I reanalyzed data in Simovich (1985). At seven of the 26 ponds, in which tadpoles were sampled in the Simovich (1985) study, the amplexed pairs of male and female adults were also sampled and individuals within the pairs identified as hybrids or pure-species types. I therefore used these data to determine if the frequency of  $F_1$  hybrid tadpoles produced was inversely correlated with the frequency of breeding *S. bombifrons* adults using a Spearman rank order correlation analysis. If hybridization was most likely when *S. bombifrons* was rare because of difficulty locating conspecific mates, then the frequency of hybrid offspring produced should be inversely related to the frequency of breeding *S. bombifrons* adults.

#### *Alternative 3: habitat changes reduce hybridization*

Because  $F_1$  hybrids produced by *S. bombifrons* females and *S. multiplicata* males are potentially favored in highly ephemeral ponds (Simovich 1985; Simovich et al. 1991; Pfennig and Simovich 2002), increases in rainfall may generate a decline in  $F_1$  hybrids if *S. bombifrons* females hybridize less often as the habitat becomes wetter. To determine whether an increase in rainfall over time contributed to the decline in hybrid frequency, I first determined if summer rainfall (June–August, the months when spadefoots breed) has increased over time. Rainfall data from Portal, Arizona, were downloaded from the U.S. National Climatic Data Center archives (<http://wfn.ncdc.noaa.gov/oa/climate/climateresources.html>). Because the data were appropriately structured and met parametric assumptions, I used regression analysis to determine if summer rainfall amounts had increased significantly over time. Such a pattern would be expected if increased summer rainfall resulted in reduced hybridization between *S. multiplicata* and *S. bombifrons*.

I next determined if the frequency of hybrid tadpoles produced in a given year was correlated with mean summer rainfall in that year, using a nonparametric Spearman rank order correlation analysis because these data did not meet parametric assumptions. I used both the estimates of hybrid tadpoles from 1999–2001 and the estimates of hybrid tadpoles from 1980–1982 reported by Simovich (1985). In this analysis, I could examine whether hybrid production is actually associated with summer rainfall, as would be expected if *S. bombifrons* females were more likely to hybridize when ponds were ephemeral, as in dry years.

## RESULTS

### *Recent Estimates of Hybrid Frequency in the Field*

The overall mean ( $\pm$  SD) frequency of  $F_1$  hybrids observed across the seven ponds sampled between 1999 and 2001 was 0.003 (0.008; see also Table 1). Yet, of these seven ponds, only one pond actually contained hybrid offspring. The relative proportion of  $F_1$  hybrid offspring in this pond was 0.022, which represented one hybrid tadpole identified of 46 tadpoles collected at this site. No pure *S. bombifrons* offspring were sampled in this pond that contained the hybrid offspring, suggesting that although at least one *S. bombifrons* had attended the breeding aggregation, it had hybridized.

### *Comparison of Recent Estimates of Hybrid Frequency with Earlier Studies*

I found a significant negative correlation between time period and estimates of  $F_1$  hybrid frequency ( $r_s = -0.494$ ,  $N = 37$ ,  $P = 0.002$ ; Fig. 2), indicating that hybrid frequency had declined over time. Additionally, when I compared the number of ponds that contained  $F_1$  hybrid offspring relative to those that did not between the two periods in which tadpoles were sampled, I found a significant decline in the proportion of ponds in which hybridization had occurred. In particular, for 1980–1982, of 26 ponds that contained both species, 21 contained  $F_1$  hybrid offspring, whereas for 1999–2001, only one of seven ponds contained hybrid offspring (log-likelihood  $\chi^2 = 10.81$ ,  $df = 1$ ,  $P = 0.001$ ).

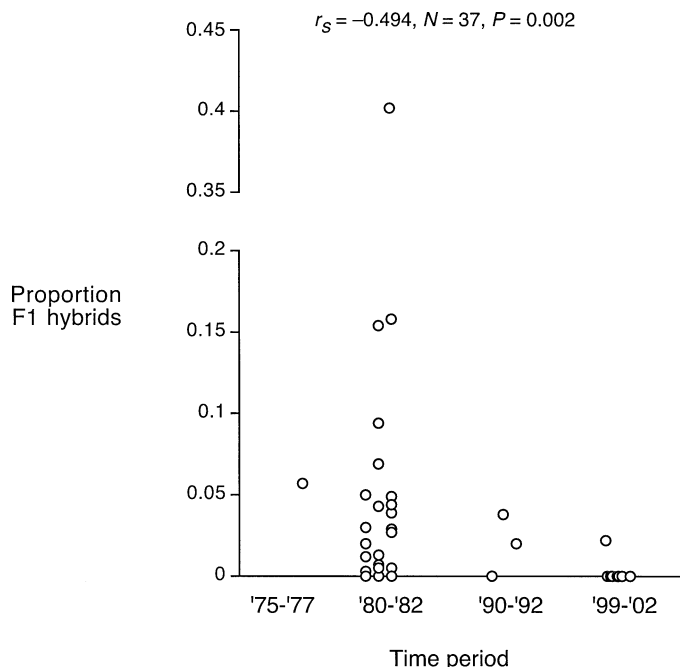


FIG. 2. Estimates of relative F<sub>1</sub> hybrid (*Spea multiplicata* × *S. bombifrons*) frequencies during four time periods. Data presented include those reported in previously published accounts (Sattler 1985; Simovich 1985; Crosswaite 1994). A nonparametric Spearman rank order correlation coefficient is reported, which is not affected by outliers present in the data. Data are offset within each time period to show overlapping points. The y-axis is split to reveal both an extreme outlier and dispersion of remaining data. Removal of the outlier does not affect the result.

Table 1 reports the means and standard errors for frequency estimates of F<sub>1</sub> hybrids and pure-species types for each time period. I found a significant effect of time period on mean estimates of F<sub>1</sub> hybrid frequency ( $H_c = 9.47$ ,  $df = 3$ ,  $P < 0.025$ ; Table 1). When I contrasted the mean estimates of F<sub>1</sub> hybrid frequency from 1999–2001 with those from 1980–1982 and 1990–1992, I found that the mean estimate of F<sub>1</sub> hybrid frequency in 1999–2001 was significantly lower than the mean estimate of hybrid frequency in 1980–1982 ( $Q = 2.81$ ,  $k = 4$ ,  $P < 0.05$ ). The mean frequency of F<sub>1</sub> hybrids was not significantly different between the time periods 1990–1992 and 1999–2001 ( $Q = 1.20$ ,  $k = 4$ ,  $P > 0.50$ ), although the mean frequency of hybrids declined by sixfold from 1990–1992 to 1999–2001. A power analysis suggested that my sample size was too small to detect significance of this effect (a minimum of 13 estimates per time period would have been required to detect significance of the observed difference in mean hybrid frequency between 1990–1992 and 1999–2001 using a *t*-test).

The above results indicate that hybridization between *S. bombifrons* and *S. multiplicata* has apparently declined over time. Below I evaluate three alternative hypotheses to reinforcement that may explain this pattern.

#### Evaluation of Alternative Hypotheses for Hybrid Decline

##### Alternative 1: opportunities for hybridization have declined

I found no evidence suggesting that the opportunities for hybridization have declined. Indeed, *S. bombifrons*, the less

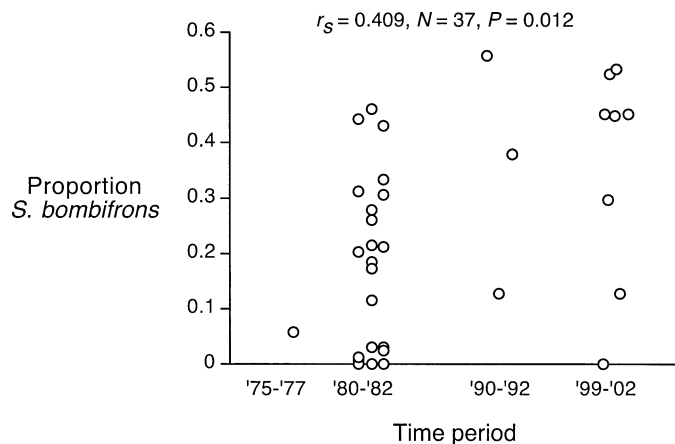


FIG. 3. Estimates of relative frequencies of the less common species, *Spea bombifrons*, during four time periods. Data presented include those reported in previously published accounts (Sattler 1985; Simovich 1985; Crosswaite 1994). A nonparametric Spearman rank order correlation coefficient is reported. Data are offset within each time period to show overlapping points.

abundant species, appears to have become more common over time (Table 1, Fig. 3). I found a significant positive correlation between the frequency of *S. bombifrons* and time ( $r_s = 0.409$ ,  $N = 37$ ,  $P = 0.012$ ; Fig. 3).

A comparison of means among the different time periods suggests that the changes in abundance of *S. bombifrons* were not as dramatic as those observed for the hybrids, however. Although the individual measures of *S. bombifrons* frequency were positively correlated with time, the mean frequency of *S. bombifrons* across the different time periods were not significantly different from one another. I found no significant effect of time period on the mean estimates of *S. bombifrons* ( $H_c = 6.55$ ,  $df = 3$ ,  $P > 0.05$ ). Moreover, contrasts of the two previous time periods with 1999–2001 revealed no significant differences among the mean estimates of *S. bombifrons* frequency (comparison of *S. bombifrons* frequency in 1980–1982 vs. 1999–2001:  $Q = 2.07$ ,  $k = 4$ ,  $P > 0.10$ ; 1990–1991 vs. 1999–2001:  $Q = 0.243$ ,  $k = 4$ ,  $P > 0.50$ ). The mean estimate of *S. bombifrons* frequency for 1990–1992 and 1999–2001 were very similar (Table 1), suggesting that the frequency of *S. bombifrons* stabilized.

These results indicate that a decline in opportunities for hybridization cannot account for the decline in hybrids over time. Indeed, although hybrid frequency declined, opportunities for hybridization appear to have actually increased over time.

##### Alternative 2: hybridization is initially common when one species is rare

The finding that the frequency of *S. bombifrons* increased over time as F<sub>1</sub> hybrid frequency declined suggests that hybridization may have been more common when *S. bombifrons* was rare because *S. bombifrons* hybridize when conspecific mates are rare, but preferentially mate with conspecifics when conspecific mates are available. Contrary to this hypothesis, however, I found that the production of F<sub>1</sub> hybrid offspring in a given aggregation was not significantly inversely cor-

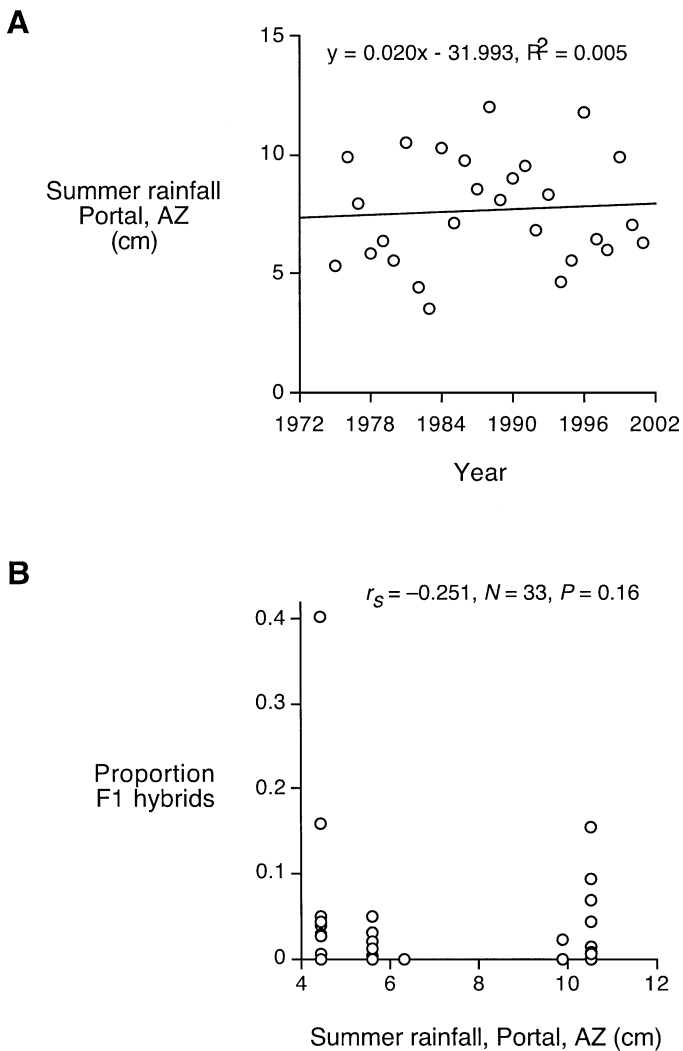


FIG. 4. (A) Summer rainfall over the 27 years during which studies were conducted measuring hybrid frequencies between *Spea multiplicata* and *S. bombifrons*. (B) Proportion of F<sub>1</sub> hybrid tadpoles versus summer rainfall. Data from Simovich (1985) are included. The outlier present in the data does not affect the nonparametric Spearman rank order correlation coefficient, and removal of the outlier from the analysis does not affect the result.

related with the relative abundance of breeding *S. bombifrons* adults attending that aggregation ( $r_s = -0.126, N = 7, P = 0.79$ ), based on a reanalysis of data in Simovich (1985).

#### Alternative 3: habitat changes reduce hybridization

I found no evidence that the frequency of hybridization between *S. multiplicata* and *S. bombifrons* was associated with changes in rainfall over time. Contrary to the hypothesis that increases in summer rainfall amount may contribute to a decline in hybrid frequency over time, average summer rainfall did not significantly increase over the years 1975 to 2001 ( $F_{1,25} = 0.122, P = 0.73$ ; Fig. 4A). Moreover, the frequency of F<sub>1</sub> tadpoles produced in a given year was not associated with average summer rainfall for that year ( $r_s = -0.251, N = 33, P = 0.16$ ; Fig. 4B).

#### DISCUSSION

The decrease in hybridization between *S. multiplicata* and *S. bombifrons* over time illustrates that the evolution of reproductive isolation between natural populations of interbreeding species is a measurable phenomenon. Moreover, because the mean frequency of hybrids declined from 5.7% to 0.3% in only 27 years, these data suggest that reproductive isolation can evolve rapidly to near completion. A population study with another species of spadefoot in the genus *Scaphiopus* suggests that 27 years may represent as few as 13 spadefoot generations (Pearson 1955). A similarly rapid decline in hybrid frequency has been reported in *Bufo* toads (Jones 1973).

Such a rapid decline in hybridization suggests that the two species may have come into contact only recently. The increase in frequency of *S. bombifrons* over time suggests that this species may have recently moved into the breeding habitats previously occupied only by *S. multiplicata*. The creation of dug-out cattle tanks by the ranching industry beginning in the late 1800s may have provided longer-lasting ephemeral ponds in which the more slowly developing *S. bombifrons* (Pfennig and Simovich 2002) was able to breed with *S. multiplicata*. Human changes to the environment that promoted contact between *S. multiplicata* and *S. bombifrons* then may have been followed by selection to avoid hybridization and the evolution of mating behaviors that precluded hybridization (i.e., reinforcement).

As I describe below, I was able to rule out alternative hypotheses other than reinforcement that could account for the observed temporal variation in hybrid frequency. However, because the population was not sampled systematically in time or space, spatial heterogeneity may also account for the observed decline in hybrid frequency if sampling locations in each study differed systematically in habitat (e.g., pond ephemerality) or the relative abundance of each species. If such spatial variation contributes to variation in hybrid frequency, the observed decline in hybrids is potentially an artifact of different sampling locations among the different time periods.

Two key points suggest that the observed decline in hybrids is not an artifact of variation in sampling locations or procedures. First, although I did not sample all the same ponds in 1999–2001 that Simovich (1985) sampled in 1980–1982, in both time periods the ponds that were sampled consisted of both small ponds (which tend to be highly ephemeral) and large ponds (which tend to be less ephemeral). All ponds sampled in both time periods were located in similar habitat and are usually separated by less than 5 km (Fig. 1). Moreover, five of the seven sites sampled in 1999–2001 were the same as or interspersed with those sampled in 1980–1982 (Fig. 1). Thus, no systematic differences in habitat or pond types are readily apparent that could contribute to different estimates in hybrid frequency between the ponds sampled in 1980–1982 and those sampled in 1999–2001.

A second point suggests that the observed decline in hybrid frequency is robust, despite the absence of systematic sampling over time. Contrary to the 1980–1982 and 1999–2001 time periods, hybrid frequency estimates in 1975–1977 and 1990–1992 were based on samples of adults. Adults were

collected along roadsides or near breeding ponds throughout the same region where the ponds sampled for tadpoles are located (Fig. 1; Sattler 1985; Crosswaite 1994). The estimates of hybrid frequency based on samples of adults are less sensitive to the problem of spatial heterogeneity, because each estimate was derived from a sampling of adults over the entire area (see Materials and Methods). Any local variation that affected hybridization rates would have been incorporated into these measures. Importantly, these measures of hybrid frequency based on adult samples are consistent in pattern with measures of hybrid frequency based on samples of tadpoles at individual breeding ponds (Table 1, Fig. 2). Thus, although additional studies are needed to examine the influence of spatial heterogeneity on the frequency of hybridization between these species, the above discussion suggests that the decline in hybridization between *S. multiplicata* and *S. bombifrons* in southeastern Arizona is genuine.

The observed decline in hybridization frequency is consistent with the reinforcement hypothesis. Yet, three hypotheses other than reinforcing natural selection against hybrids can also explain the decline in hybridization between *S. multiplicata* and *S. bombifrons*. These hypotheses were not supported, however.

The first alternative hypothesis that I was able to reject predicted that opportunities for hybridization may have declined as a consequence of one species becoming increasingly rare (see introduction). However, the rarer of the two species, *S. bombifrons*, has become increasingly common over time. Thus, opportunities for hybridization actually increased over the same period of time during which hybridization was observed to decrease (cf. Figs. 2 and 3; see also Table 1).

The second alternative hypothesis predicted that the rarer species, *S. bombifrons*, may hybridize when conspecific mates are rare or unavailable but mate preferentially with conspecifics when conspecific mates are available. In other words, *S. bombifrons* may modify their behavior depending on the availability of conspecific mates at a given breeding aggregation. This hypothesis received some support by the finding that *S. bombifrons* has become increasingly common over time (see above).

However, I was able to reject the second alternative hypothesis for two reasons. First, I found no support for the critical prediction that *S. bombifrons* were more likely to hybridize when rare but less likely to hybridize when common. Specifically, I found that the production of F<sub>1</sub> hybrid tadpoles was not significantly correlated with the frequency of *S. bombifrons* adults at a breeding event. If *S. bombifrons* are more likely to hybridize when conspecifics are rare, then I should have found an inverse correlation between the frequency of breeding adults and the frequency of hybrids that were produced.

A second line of evidence suggesting that the decline in hybridization was not caused by increases in the frequency of *S. bombifrons* is that the frequency of *S. bombifrons* appeared to stabilize around 35%, while the frequency of hybrids continued to decline (Table 1). Indeed, although the mean frequency of *S. bombifrons* during 1999–2001 was just 1.04 times smaller than the mean estimate of *S. bombifrons* frequency during 1990–1992, the mean estimate of F<sub>1</sub> hybrids was 6.33 times smaller in 1999–2001 than in 1990–1992

(Table 1). If the decline in hybrids was caused by increases in the frequency of *S. bombifrons*, I should not have found such a pattern. Taken together with the finding that the frequency of hybrid tadpoles was not inversely correlated with the frequency of breeding *S. bombifrons* adults, the above results indicate that the observed decline in the frequency of hybrids was not caused by *S. bombifrons* hybridizing when conspecifics were rare but not when conspecifics were common.

Finally, I found no support for the third alternative hypothesis, which posited that increases in rainfall contributed to the decline in hybridization. In particular, *S. bombifrons* females may benefit by hybridizing in dry years when ponds are most ephemeral (Pfennig and Simovich 2002). Thus, if rainfall increased over the 27 years during which hybrids were sampled, then natural selection might have favored a decrease in adaptive hybridization by *S. bombifrons*.

I was able to reject the third alternative hypothesis for two reasons. First, rainfall did not increase significantly throughout the 27 years during which hybrids were observed to decline. Second, although *S. bombifrons* females may benefit by hybridizing in dry years when ponds are most ephemeral (Pfennig and Simovich 2002), I found no relationship between the frequency of hybrids produced and summer rainfall. The decline in hybrids over time therefore does not appear to result from *S. bombifrons* females hybridizing less often because of changes in rainfall over time.

Although I found little support for the three alternative hypotheses described above, two lines of evidence strongly suggest that *S. bombifrons* and *S. multiplicata* may be undergoing reinforcement. First, natural selection appears to select against hybridization: F<sub>1</sub> hybrids generally have reduced fitness relative to parental types (Simovich 1985; Simovich et al. 1991; Pfennig and Simovich 2002). Finding selection against hybrids provides a mechanism by which reproductive isolating mechanisms might be favored to evolve. Second, and perhaps as a consequence of natural selection against the production of hybrids, mating behaviors in sympatry that preclude hybridization appear to have evolved (Pierce 1976; Pfennig 2000; K. Pfennig, unpubl. data). Generally, selection against hybrids in conjunction with behaviors in sympatry that preclude hybridization are hallmarks of reinforcement (Dobzhansky 1940; reviewed in Blair 1974; Howard 1993; Butlin 1995). The exhibition of these characteristics by *S. bombifrons* and *S. multiplicata* suggest that they may be undergoing reinforcement.

The finding that F<sub>1</sub> hybrids have declined over time is in accord with the expectation that reinforcement will promote the evolution of reproductive isolation between hybridizing species (Dobzhansky 1940; Jones 1973; Blair 1974; Britch et al. 2001). These results are therefore consistent with the hypothesis that natural selection to prevent the production of unfit hybrids can enhance and perhaps even finalize reproductive isolation between hybridizing species. The finding that F<sub>1</sub> hybrids have come to be so rare in as little as 27 years is particularly striking and suggests that reinforcing natural selection against hybrids may be a potent force generating reproductive isolation between recently diverged populations or species. Indeed, reinforcement that is not impeded by countervailing factors such as gene flow may generate rapid



divergence. Consequently, many systems that have undergone reinforcement may be overlooked because reproductive isolation between the interacting populations or species may already be complete.

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