DIFFERENTIAL SELECTION TO AVOID HYBRIDIZATION IN TWO TOAD SPECIES

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Abstract.—The fitness consequences of hybridization critically affect the speciation process. When hybridization is costly, selection favors the evolution of prezygotic isolating mechanisms (e.g., mating behaviors) that reduce heterospecific matings and, consequently, enhance reproductive isolation between species (a process termed reinforcement). If, however, selection to avoid hybridization differs between species, reinforcement may be impeded. Here, we examined both the frequency and fitness effects of hybridization between plains spadefoot toads (*Spea bombifrons*) and New Mexico spadefoot toads (*S. multiplicata*). Hybridization was most frequent in smaller breeding ponds that tend to be ephemeral, and heterospecific pairs consisted almost entirely of *S. bombifrons* females and *S. multiplicata* males. Moreover, in controlled experimental crosses, hybrid offspring from crosses in which *S. multiplicata* was maternal had significantly lower survival and longer development time than pure *S. multiplicata* offspring. By contrast, hybrid offspring from crosses in which *S. bombifrons* was maternal outperformed pure *S. bombifrons* offspring by reaching metamorphosis faster. These data suggest that, although *S. multiplicata* females are under selection to avoid hybridization, selection might favor those *S. bombifrons* females that hybridize with *S. multiplicata* if their breeding pond is highly ephemeral. Generally, the strength of selection to avoid hybridization may differ for hybridizing species, possibly impeding reinforcement.

Key words.—Adaptive hybridization, female mate choice, hybrid fitness, introgression, reinforcement, speciation, *Spea*.

Speciation by reinforcement is a potentially important means by which populations become reproductively isolated. When hybridization by two sympatric species produces offspring that have lower fitness than pure-species types, selection can lead to the evolution of premating isolating barriers (e.g., mating behaviors) that reduce hybridization (Howard 1993; Gerhardt 1994; Noor 1995; Coyne and Orr 1997; Saetre et al. 1997; Higgie et al. 2000; Pfennig 2000). Consequently, the evolution of premating barriers in response to costly hybridization can enhance reproductive isolation further between interbreeding populations, a process termed reinforcement (Dobzhansky 1940; Howard 1993; Butlin 1995; Noor 1999).

Low hybrid fitness is generally considered a prerequisite for reinforcement (Spencer et al. 1986; Liou and Price 1994; Butlin 1995; but see Kirkpatrick and Servedio 1999). Although reduced hybrid fitness relative to pure-species types is common (reviewed in Barton and Hewitt 1989; Howard 1993), hybridization can sometimes be beneficial (reviewed in Arnold and Hodges 1995; Arnold 1997; see also Semlitsch and Reyer 1992; Semlitsch 1993; Burke et al. 1998; Parris 1999, 2001a; Veen et al. 2001). Moreover, the fitness consequences of hybridization may vary across environments or fitness components (Semlitsch and Reyer 1992; Emms and Arnold 1997; Wang et al. 1997; Burke et al. 1998; Pearson 2000; Parris 2001a,b). For instance, hybrids may outperform pure-species types early in ontogeny but may suffer lower fecundity as adults (e.g., Simovich 1985; Simovich et al. 1991; Parris et al. 1999). Thus, to evaluate the fitness effects of hybridization, and consequently, the strength of selection on premating barriers to hybridization, we must consider how interactions of different fitness components result in a net advantage or disadvantage for hybrids in different environmental contexts.

An additional complicating factor in studies of hybrid fitness is that the strength of selection to avoid hybridization may differ for each of the hybridizing species (Parris et al. 1999; Pearson 2000; Pearson and Rohwer 2000; Tiffin et al. 2001; Veen et al. 2001). For example, hybrid fitness may be intermediate between parental types (Parris et al. 1999; Pearson 2000; Pearson and Rohwer 2000), such as when traits associated with fitness exhibit additive genetic variance. In such cases, selection may favor hybridization by one species but not the other. Selection to avoid hybridization also will differ between interacting species when the fitness of hybrid offspring produced from crosses in which one species is maternal differs from the fitness of hybrid offspring produced from crosses in which the alternative species is maternal. That selection to avoid hybridization can vary between interacting species is not generally considered in theoretical investigations of reinforcement, but such a pattern of selection may affect whether and how reinforcement proceeds. Indeed, if selection favors hybridization by one species, then sufficient introgression may result to impede reinforcement (sensu Kelly and Noor 1996; Servedio and Kirkpatrick 1997; see also Tiffin et al. 2001).

Here we present data characterizing both the frequency and fitness effects of hybridization between plains spadefoot toads, *Spea bombifrons*, and New Mexico spadefoot toads, *S. multiplicata*. We examine the circumstances under which hybridization occurs in the field and evaluate the possibility that the strength of selection to avoid hybridization differs for females of the two species.

Spea multiplicata and *S. bombifrons* coexist in the southwestern United States. These species are the most divergent species within the *Spea* genus (Wiens and Titus 1991). Sev-

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eral aspects of the spadefoot toad system make them ideal candidates for studying hybridization and its role in reinforcement. First, in southeastern Arizona, adult F_1 hybrids suffer reduced fitness relative to pure-species types: Male hybrids are sterile and female hybrids have reduced fecundity (Simovich 1985; Simovich et al. 1991). The fitness of other hybrid types is unknown. Second, *S. multiplicata* females select against conspecific males that resemble heterospecifics in sympatry but not allopatry, suggesting that selection to avoid costly hybrid matings has resulted in the evolution of mating behaviors that reduce hybridization between these species (Pfennig 2000). Third, male *S. bombifrons* produce calls that are more dissimilar to those of *S. multiplicata* where the two species coexist than where *S. multiplicata* is absent (Pierce 1976).

Although spadefoots exhibit the hallmarks of reinforcement (e.g., low hybrid fitness and character displacement in both female and male mating behaviors), two issues remain unresolved. First, hybridization may be favored in some environmental contexts, but not in others. In particular, Simovich and colleagues (Simovich 1985, 1994; Simovich et al. 1991) showed that in both experimental contexts and in the field, hybrid tadpoles in which *S. bombifrons* was maternal can develop faster than pure-species *S. bombifrons* tadpoles and could therefore be favored in highly ephemeral ponds. Thus, *S. bombifrons* females that hybridize might be selectively favored in ephemeral pools. Such a pattern of selection could enhance introgression rather than reinforcement. Evaluating whether hybridization might not be selected against in some circumstances is therefore critical to assessing the conditions under which reinforcement can occur. Second, observations at natural breeding aggregations (see results below and Simovich 1985) suggested that hybridization between *S. bombifrons* females and *S. multiplicata* males was more likely than pairings between *S. multiplicata* females and *S. bombifrons* males. One evolutionary explanation for this pattern is that selection to avoid hybridization differs between the two species.

The goals of the present study were twofold. First, we used field observations to evaluate the circumstances under which hybridization occurred and assessed whether females of one species were more likely to mate with heterospecifics. Second, we experimentally crossed the two species and reared the offspring of these crosses in experimental wading pools to evaluate the potential fitness effects of hybridization for females of each species. By doing so, we could assess whether the strength of selection to avoid hybridization potentially differs for females of each species.

Materials and Methods

Field Observations of Hybridization

We examined patterns of hybridization in nature using field collections and observations. We used these data to address how frequent hybridization was, and whether hybridization was more likely to occur in some breeding aggregations than others. Second, we examined whether males or females of one species hybridize more often than males or females of the other species. We used data on hybrid frequency of tadpoles in natural breeding ponds, pond duration, and incidence

of heterotypic matings that one of us (MAS) collected from 1980 to 1982.

To estimate frequency of hybrid larvae, early tadpoles (approximately 7–12 days of age) were collected by dip-net from throughout each of 27 ponds following 39 independent breeding events (i.e., some ponds were sampled in multiple years or multiple times within a year if it dried completely and refilled, thereby causing another breeding event). The mean number of tadpoles sampled per pond was 158. The minimum number of tadpoles sampled in a pond was five; this pond was located in an area that *S. bombifrons* does not inhabit, and no hybrids were detected at this site. The maximum number of tadpoles sampled in a pond was 737.

Using four diagnostic allozyme markers (Simovich and Sassaman 1986), tadpoles were identified as pure species *S. multiplicata* (M) or *S. bombifrons* (B); F_1 hybrids (H); offspring of crosses between F_1 hybrids and *S. multiplicata* (BKM); offspring of crosses between F_1 hybrids and *S. bombifrons* (BKB); and offspring of generations of mixing, which were designated double-backcrosses (DBK). The use of four independent markers underestimates the frequency of backcrosses (Simovich and Sassaman 1986), so the estimates of relative frequency of these classes are conservative.

The surface area (SA) of each pond was determined from pond measurements following each sampling event. Pond size varies with rainfall amount, so size is not necessarily fixed from one breeding aggregation to the next or one year to the next. The ponds were separated into five size classes: (1) < 250 m²; (2) > 250–500 m²; (3) > 500–1000 m²; (4) > 1000–1500 m²; and (5) > 1500 m². Pond SA is correlated positively with pond depth (Simovich 1985), and provides an indication of how rapidly a pond may dry: Ponds with smaller SA dry more quickly than do those with larger SA.

Additionally, the distribution of adult types and incidence of heterotypic pairing was measured among adults at nine breeding aggregations in four of the above ponds over the three-year period. Ponds chosen for this analysis were those in which hybrid offspring were known to occur.

Breeding adults were sampled from the ponds by collecting amplexed pairs. Tissue was collected by toe-clip from each member of the pair. The pair was then released without breaking up amplexus so that breeding was not disrupted. Sampling concluded at dawn or after 50 pairs had been collected, whichever came first. No pair was sampled more than once, and the collections typically included 75–80% of the pairs observed in each pond. Each adult from the amplexed pairs was classified according to its species by employing the methods described above for the tadpoles. Unmated animals from the breeding aggregation were not sampled. The number of calling males was estimated by counting calling males at eight of the aggregations. Pond area was also determined, so that male density was determined for each of these aggregations.

In addition to those pairings of adults observed as above, we also report the number of mismatches between *S. multiplicata* and *S. bombifrons* observed during 1995–2000. For these pairings, one of us (KSP) identified the species of each adult by using morphology, which is a reliable indicator of species identity (Simovich 1985; Simovich and Sassaman 1986).

Experimental Crosses

To examine the fitness consequences of hybridization in the larval stage for females of each species, one of us (KSP) collected amplexed pure-species pairs of *S. multiplicata* and *S. bombifrons* from a single natural breeding aggregation in 2000 near Portal, Arizona. Identification of species was based on morphology, which can be used to assess species identity of adults (Simovich 1985; Simovich and Sassaman 1986). The adults were returned immediately to the laboratory (Southwestern Research Station, Portal, AZ), where each collected pair was randomly assigned for use in a pure-species pairing or for use in hybridization. The pairs to be used for hybridization were then randomly assigned to six sets of two amplexed pairs each. The pairs in each of these sets were separated, and the male of the first pair in a set was placed with the female of the second pair from the same set; the female of the first pair was placed with the male from the second pair. Consequently, 12 mixed-species pairs were created, half of which had *S. multiplicata* as the female and half of which had *S. bombifrons* as the female. Such a design permits assessing both the overall performance of hybrid offspring relative to pure species types as well as the fitness effects of hybridization for females of each species.

Those pairs not used for hybridization were kept together as pure-species pairs. Of the pairs collected at the breeding aggregation, 13 were maintained as pure *S. multiplicata* pairs, and eight were maintained as pure *S. bombifrons* pairs. In addition to these experimental crosses, two naturally occurring mixed-species pairs were collected from the same breeding aggregation as those above. In both pairs, *S. bombifrons* was the female and *S. multiplicata* was the male. These pairs were not separated; instead, each pair was kept together to produce two additional sibships of hybrid offspring with *S. bombifrons* as the female.

Each of the above pairs was placed in a tank containing 13.5 L dechlorinated water and left overnight. All but seven pairs oviposited. Thus, the final number of pairs contributing offspring to each treatment group was nine pure-species *S. multiplicata* pairs (MM), eight pure-species *S. bombifrons* pairs (BB), five mixed-species pairs in which *S. multiplicata* was the female (MB), and six mixed-species pairs in which *S. bombifrons* was the female (BM). Following oviposition, the adults were removed and the eggs aerated until the tadpoles were free swimming (about two days after oviposition).

Once the tadpoles were free swimming, 16 tadpoles from each clutch were randomly selected and transferred to tanks filled with 6 L dechlorinated water to avoid differential density effects on early growth of tadpoles. Three days later, eight tadpoles were removed at random from these groups of 16 and transferred to cages in outdoor wading pools (see below).

Tadpoles were reared to metamorphosis in cylindrical mesh cages placed in 1.8-m diameter wading pools that were allowed to dry naturally over the course of the experiment. Although the rearing pools did not replicate field conditions exactly, allowing the pools to dry provided the tadpoles with a critical cue (pond drying) that can trigger metamorphosis. Metamorphosis in response to pond drying is important, because tadpoles do not survive when a pond dries. Initially, the water in each pool was approximately 23 cm deep. Eight tadpoles from the same sibship were placed in each of 28 cages within each pool. All sibships were represented in each of the four pools.

The wading pools were placed in a square array approximately 1 m apart. The cages within the pools were mesh cylinders (18 cm diameter × 28 cm high) constructed of aluminum screening. The bottom of the cylinder was also aluminum mesh that was sealed to the sides of the cylinder with silicone. The top was pinched closed to exclude predators but provide access for feeding and metamorph removal. One cage containing MM offspring was lost to raccoon predation. The cages were arranged in the pools so that the hybrid and pure-species sibships were spread throughout the pool. The cages were ordered so that the same treatments were never adjacent. Within this constraint of ordering treatments, the sibships were randomly assigned locations in the first pool. The ordering of the sibships was then maintained in all four pools, but their placement in a pool was shifted by a 90° rotation of the cages in each of the subsequent three pools so that no sibship was consistently placed in the same relative location (e.g., the west side) of every pool. Finally, each cage was individually numbered so that all censuses and metamorph measurements were blind with respect to the treatment group.

Tadpoles were censused throughout the experiment and fed high-protein fish food ad libitum. Tadpoles were not fed natural live food (e.g., fairy shrimp), because doing so can induce the production of carnivore-morph tadpoles, which potentially cannibalize other tadpoles (reviewed in Pfennig and Murphy 2000). Live food can enhance development times of spadefoots (Simovich 1985; Simovich et al. 1991), however, so our measures of development times on fish food are potentially conservative (see Discussion for comparison of our results to other studies). Cages were checked daily until the first metamorph appeared. After this point, all cages were checked twice daily (morning and evening) and any metamorphs in the cages were immediately removed. For each tadpole, the date and general time (i.e., A.M. or P.M.) of metamorphosis was recorded, with metamorphosis defined as the time at which a first forelimb had emerged. After removal from its cage, each metamorph was immediately measured for snout-vent length and mass (hereafter referred to as ''raw mass'').

At 35 days following breeding, the experiment was terminated and the number of tadpoles remaining in each cage was counted. The wading pools were almost completely dry by this point (the remaining water in each pool was less than approximately 2 cm deep), and nearly all tadpoles (92% of total animals surviving at end of experiment) had metamorphosed.

Because individuals within a cage were not independent, we calculated the mean growth measures of all metamorphs from each cage to obtain a single measure per cage for age, raw mass, and snout-vent length (SVL) at metamorphosis. Additionally, for each cage we measured total survival per cage as the number of metamorphs removed from the cage plus the tadpoles (if any) remaining in the cage at the end of the experiment.

Prior to analysis, we log-transformed age, SVL, and raw

TABLE 1. Proportion of pure-species and hybrid types among spade-foot tadpoles sampled at natural breeding ponds. Included are ranges of proportions observed, means with standard deviations, and number of ponds in which type observed (of 39 sampled). See text for type definitions.

| Type	Proportion		N
	Range	Mean ± SD	
M	0.242–1.000	0.844 ± 0.190	39
B	0.005–0.460	0.200 ± 0.152	20
H	0.003–0.402	0.049 ± 0.084	21
BKM	0.008–0.103	0.035 ± 0.030	18
BKB	0.005–0.050	0.020 ± 0.015	7
DBK	0.003–0.010	0.007 ± 0.004	3

FIG. 1. Frequency of participation in heterotypic matings by sex and species. Sb, *Spea bombifrons*; Sm, *Spea multiplicata*. Numbers in parentheses show sample sizes.

mass at metamorphosis to meet the assumptions of parametric analyses. Transformation did not normalize our survival data, however, so we used nonparametric analyses for these data (see below). Because raw mass was highly correlated with SVL ($r = 0.94$, $P < 0.001$, $N = 111$), we controlled for SVL in our measure of mass by computing the residuals of the cubic regression of mass (log-transformed) on SVL (log-transformed). These residuals are hereafter referred to as ''standardized mass'' and provide an indication of condition at metamorphosis. We report means and standard errors for raw mass among the different treatment groups (see Results), but all analyses of treatment effects were performed on our standardized mass measure.

We used ANOVA to determine if there was a treatment effect on age, SVL, and standardized mass at metamorphosis. Rearing pool did not effect age at metamorphosis, so we removed the rearing pool effect from our analysis of treatment effect on age at metamorphosis and combined data across the pools. In all other analyses, the pools were treated as blocks and were included in the analyses as a random effect. Additionally, because both SVL and standardized mass can vary with age at metamorphosis, we used ANCOVA in which age at metamorphosis was included as a covariate to control for the effect of metamorphic age on these growth measures. Following these analyses, we performed Tukey tests for unequal sample sizes to detect differences among the groups (Sokal and Rohlf 1995; Zar 1999).

Finally, because the survival data did not meet the assumptions of parametric analyses, we analyzed these data using a Kruskal-Wallis test with tied ranks (survival did not differ significantly among the rearing pools, so the data were combined across blocks; Sokal and Rohlf 1995; Zar 1999). We found a significant treatment effect in this analysis, so we used a nonparametric multiple comparisons test for unequal sample sizes and tied ranks to evaluate differences among the treatment groups (Zar 1999).

RESULTS

Field Observations of Hybridization

Of the 39 breeding aggregations where tadpoles were sampled, 24 contained tadpoles of mixed species genotypes. F_1 hybrid types were detected in five ponds where no *S. bombifrons* offspring were collected, indicating that *S. bombifrons* adults had been present at the breeding aggregations, but had
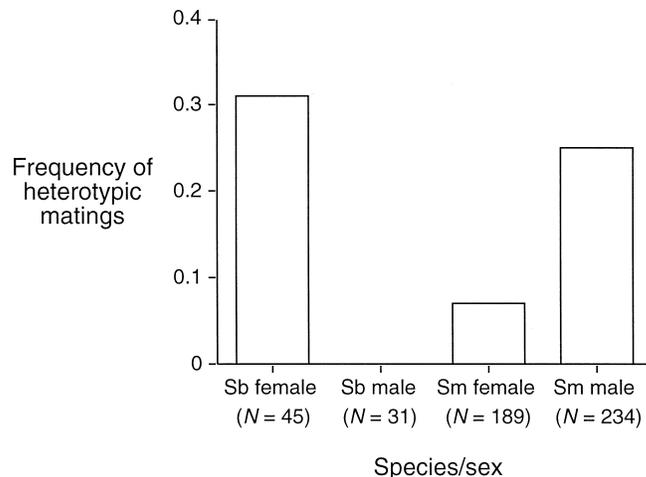
hybridized. Of the 20 ponds sampled that contained pure *S. bombifrons* offspring, five contained no F_1 hybrid offspring, suggesting that although *S. bombifrons* and *S. multiplicata* adults coexisted at these breeding aggregations, no hybridization took place. In those ponds where F_1 hybrid offspring did occur, their relative frequency varied from 0.3% to 40% of the tadpoles sampled (Table 1).

The presence of backcross offspring suggested that introgression had occurred. Backcross offspring to *S. multiplicata* (BKM) were found in more ponds than backcross offspring to *S. bombifrons* (BKB; log-likelihood $\chi_1^2 = 5.01$, $P = 0.025$; Table 1). However, the mean relative frequency of BKM offspring was not significantly different from that of the BKB offspring (Wilcoxon rank sum: $S = 69$, $Z = -1.31$, $P = 0.19$; Table 1).

When we examined the relationship between F_1 hybrid frequency and pond size, we found that the relative frequency of F_1 hybrids was significantly, negatively correlated with pond SA (Spearman's rank-order correlation coefficient, $r_s = -0.40$, $P = 0.04$, $N = 26$), indicating that hybridization occurred more often in smaller ponds that dry more rapidly. Additionally, the frequency of crosses between *S. multiplicata* and *S. bombifrons* adults was correlated with male density ($r_s = 0.78$, $P = 0.02$, $N = 8$), indicating that hybridization is also associated with male density. Although male density can potentially be associated with pond SA, we did not find a statistically significant correlation between male density and pond SA ($r_s = -0.42$, $P = 0.30$, $N = 8$). Because our sample size for this comparison was small, our power to detect a significant correlation may have been too low.

When we examined the frequency with which each sex of each species engaged in heterotypic matings across all ponds, we found that the likelihood of engaging in any heterotypic pairing varied significantly among sex and species (log-likelihood $\chi_3^2 = 41.91$, $P < 0.001$; Fig. 1). *Spea bombifrons* females and *S. multiplicata* males engaged in heterotypic matings more often than *S. bombifrons* males and *S. multiplicata* females (Fig. 1, Table 2). Indeed, 10 pairs consisting of *S. bombifrons* females mated to *S. multiplicata* males were ob-

TABLE 2. Mating types observed at natural breeding aggregations. Proportions of all matings and proportion of each mating type within the categories of homotypic matings and heterotypic matings. Female type given first followed by male type.

		Proportion of	
Mated pair	N	All matings	Within category
Homotypic pairs			
M × M	175	0.549	0.729
B × B	31	0.097	0.129
H × H	34	0.107	0.142
Heterotypic pairs			
H × M	46	0.144	0.582
M × H	13	0.041	0.165
B × H	2	0.006	0.025
B × M	10	0.031	0.127
M × BKM	1	0.003	0.013
BKM × M	3	0.009	0.038
H × BKM	2	0.006	0.025
B × BKB	1	0.003	0.013
B × DBK	1	0.003	0.013

served across all ponds sampled, whereas no pairs involving *S. multiplicata* females with *S. bombifrons* males were observed (Fig. 1, Table 2). Similarly, during 1995–2000, 10 pairs consisting of *S. bombifrons* females with *S. multiplicata* males were observed, whereas only one pair consisting of a *S. multiplicata* female with a *S. bombifrons* male was observed. These data, in conjunction with those showing that BKM offspring exist in more ponds, suggest that introgression may be primarily unidirectional into *S. multiplicata*.

Experimental Crosses

The general finding from the offspring rearing experiment indicated that the fitness effects of hybridization differ for the two interacting species (see Fig. 2). The effect of treatment on survival was significant ($H_c = 11.72$, df = 3, $p < 0.01$). Offspring produced from MB crosses (*S. multiplicata* females with *S. bombifrons* males) had significantly lower survival than MM offspring (pure *S. multiplicata*; $Q = 2.79$, $P < 0.05$) or BB offspring (pure *S. bombifrons*; $Q = 3.13$, $P < 0.02$). By contrast, offspring produced from BM crosses (*S. bombifrons* females with *S. multiplicata* males) were not significantly different in survival from the BB offspring ($Q = 1.68$, $P > 0.50$). The two hybrid types (BM and MB) did not differ in survival ($Q = 1.45$, $P > 0.50$). Additional comparisons were inappropriate because of nonsignificant differences detected above.

As with survival, the effect of treatment was significant on age at metamorphosis ($F_{3,107} = 37.94$, $P < 0.001$). When we compared age at metamorphosis among the different treatment groups, we found that all but the two hybrid groups differed. BB offspring metamorphosed at a significantly older age than the MM, BM, and MB groups (respectively, $q_{107,4} = 14.74$, $P < 0.001$; $q_{107,4} = 9.09$, $P < 0.001$; $q_{107,4} = 5.29$, $P < 0.005$). MB offspring metamorphosed at a later age than the MM offspring ($q_{107,4} = 7.48$, $P < 0.001$), but there was no difference in age at metamorphosis between the MB and BM offspring ($q_{107,4} = 3.13$, $P > 0.05$). The BM offspring metamorphosed at a significantly older age than the MM

offspring ($q_{107,4} = 4.34$, $P < 0.025$). Thus, for age at metamorphosis, hybrids were intermediate in phenotype between the parental types (Fig. 2).

The effect of treatment was also significant on both SVL and standardized mass at metamorphosis (Tables 3, 4). As with age at metamorphosis, hybrids tended to be intermediate in SVL between the parental species types (Fig. 2). When the effect of age at metamorphosis was not controlled, MM offspring achieved metamorphosis at a smaller size than BB, BM, and MB offspring (Table 3). BB offspring, by contrast, were not significantly different in size from offspring of either hybrid class (Table 3).

When we used ANCOVA to control for the effect of age on SVL at metamorphosis, we found similar results to those above. A comparison of least square means of SVL at metamorphosis (which controlled for age at metamorphosis) among the offspring treatment groups revealed that, for a given age, BB offspring reached metamorphosis at a larger SVL than both the BM and MM offspring (Table 4), but BB and MB offspring were not significantly different in their SVL at metamorphosis (Table 4). By contrast, MM offspring reached metamorphosis at a significantly smaller SVL than either the MB or BM offspring (Table 4). Moreover, when age is controlled, BM offspring reached metamorphosis at a significantly smaller SVL than did MB offspring (Table 4).

In comparing mean standardized mass (without age controlled) among treatment groups, we found that BM offspring were significantly heavier for a given body size at metamorphosis than either BB or MB offspring (Table 3). No other groups differed in standardized mass when age was not controlled. However, a comparison of least square means of standardized mass at metamorphosis (which controlled for age at metamorphosis) among the offspring treatment groups revealed that, for a given age, BM offspring were significantly heavier for a given body size at metamorphosis than the MM offspring (Table 4). No other groups were significantly different (Table 4).

DISCUSSION

Under most models of speciation by reinforcement, costly hybridization favors avoidance of heterospecific mates (e.g., Liou and Price 1994; Kirkpatrick and Servedio 1999). Yet, the possibility that each species in the interaction might be under different selection pressures to avoid hybridization has generally not been considered (but see review in Tiffin et al. 2001). Our results suggest that the strength of selection to avoid hybridization by female spadefoots may differ between species.

Hybridization is apparently always costly for *S. multiplicata* females. Although in the context of this experiment matings between *S. multiplicata* females and *S. bombifrons* males produced offspring that were larger than pure *S. multiplicata* offspring, 11% fewer MB offspring survived to metamorphosis. Moreover, MB offspring developed more slowly and would therefore be less likely to survive a drying pond than pure *S. multiplicata*. Finally, because male hybrids are sterile and female hybrids are less fecund (Simovich 1985; Simovich et al. 1991), selection on *S. multiplicata* females to avoid hybridization should be strong.
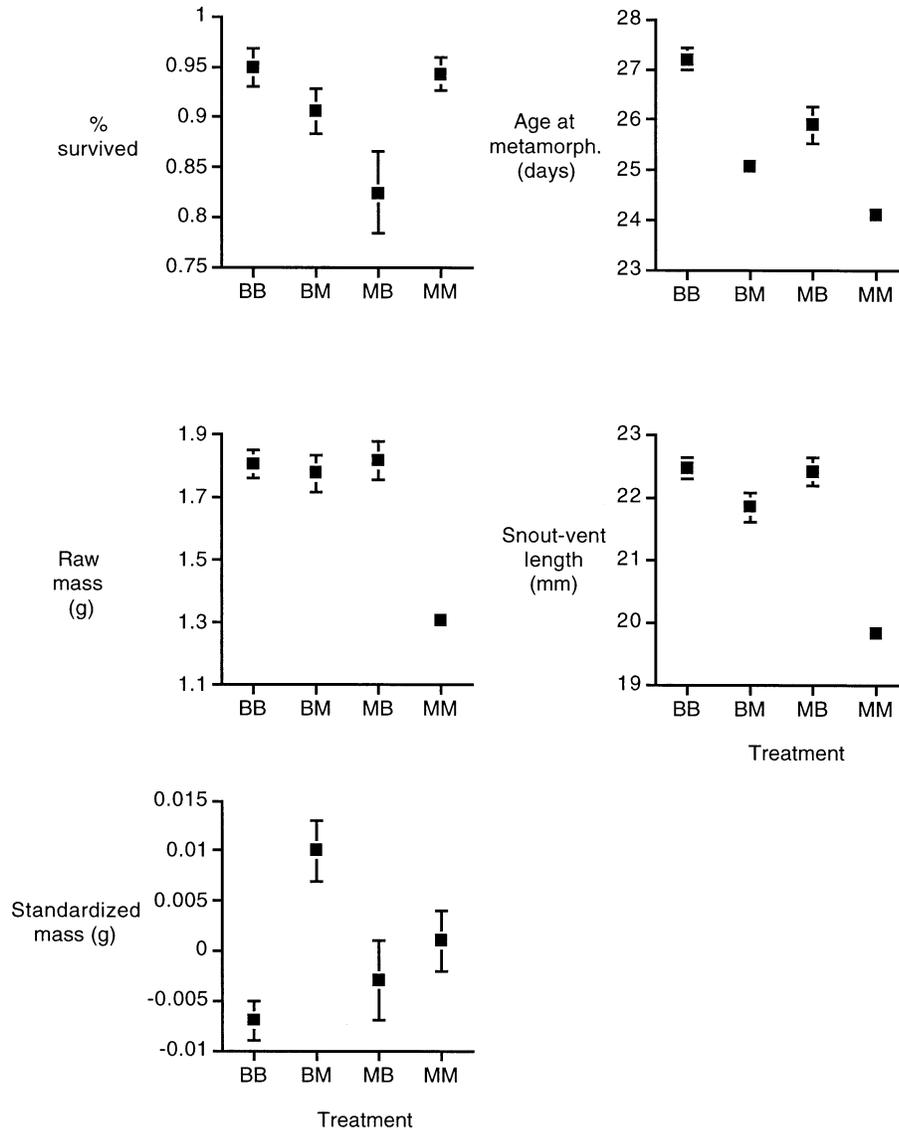
Fig. 2. Means (± SEM) among treatment groups for survival and age, raw mass, snout-vent length, and standardized mass at meta-morphosis. See text for method of deriving standardized mass. Snout-vent length, raw mass, and age at metamorphosis were back-transformed from a log scale. BB, crosses in which both parents were *Spea bombifrons*; BM, hybrid crosses with *S. bombifrons* maternal; MB, hybrid crosses with *S. multiplicata* maternal; MM, crosses where both parents were *S. multiplicata*.

The mating behaviors of *S. multiplicata* females appear to reflect a pattern of selection to avoid hybridization. Contrary to their preferences in allopatry, *S. multiplicata* females in sympatry with *S. bombifrons* select against conspecific mates that resemble heterospecifics (Pfennig 2000). Moreover, no *S. multiplicata* females were observed in the field paired with *S. bombifrons* males during 1980–1982 (Table 2), and only one of 10 hybrid pairs consisted of a *S. multiplicata* female with a *S. bombifrons* male during 1995–2000.

In contrast to *S. multiplicata* females, those *S. bombifrons* females that hybridize may not necessarily be selected against in some circumstances (Fig. 2). Although hybrid offspring produced by *S. bombifrons* females in this experiment tended to be both smaller at metamorphosis and had on average 4% lower survival than pure *S. bombifrons*, BM offspring developed faster and were heavier for a given body size at metamorphosis than pure *S. bombifrons* offspring (although for a given age the two groups did not differ in standardized mass). These results indicate that in the larval phase, BM tadpoles can potentially outperform pure *S. bombifrons* tadpoles in at least one critical fitness component: development time. Previous studies (Simovich 1985; Simovich et al. 1991) have also shown that BM offspring reach metamorphosis before BB offspring. In these experiments, tadpoles were reared on diets of fish food and a natural diet of live fairy shrimp. Moreover, in a field study, Simovich (1985, 1994) found that hybrid tadpoles (most likely BM crosses based on observations of adult pairings (see Table 2, Fig. 1) reached metamorphosis before pure *S. bombifrons* offspring, suggesting that BM tadpoles also outperform BB tadpoles in the field. Taken together, these studies indicate that the performance of BM offspring relative to BB offspring is robust to

TABLE 3. Analyses of variance and Tukey tests for the effects of hybridization treatment on (A) snout-vent length at metamorphosis and (B) standardized mass at metamorphosis. Pool, rearing pool effect. Snout-vent length and mass were log transformed; see text for derivation of standardized mass.

A. Snout-vent length at metamorphosis

Source of variation	df	SS	F	P
Treatment	3	0.056	64.804	<0.001
Pool	3	0.005	5.303	0.002
Residual	104	0.030		

Samples ranked by mean	MM	BM	MB	BB
Ranked means	1.319	1.358	1.369	1.370
Size of samples	35	24	20	32

Comparison	Difference	SE	q	P
BB vs. MM	0.052	0.003	17.530	<0.001
BB vs. BM	0.012	0.003	3.675	>0.05
MB vs. MM	0.050	0.003	14.905	<0.001
BM vs. MM	0.040	0.003	12.434	<0.001

B. Standardized mass at metamorphosis

Source of variation	df	SS	F	P
Treatment	3	0.004	5.235	0.002
Pool	3	0.004	5.788	0.001
Residual	104	0.026		

Samples ranked by mean	BB	MB	MM	BM
Ranked means	−0.007	−0.003	0.001	0.010
Size of samples	32	20	35	24

Comparison	Difference	SE	q	P
BM vs. BB	0.016	0.003	5.458	<0.005
BM vs. MB	0.013	0.003	3.746	<0.05
BM vs. MM	0.009	0.003	3.134	>0.10
MM vs. BB	0.007	0.003	2.630	>0.20

TABLE 4. Analyses of covariance and Tukey tests for the effects of hybridization treatment on (A) snout-vent length at metamorphosis and (B) standardized mass at metamorphosis. Age, age at metamorphosis; Pool, rearing pool effect. Snout-vent length and mass were log transformed; see text for derivation of standardized mass.

A. Snout-vent length at metamorphosis

Source of variation	df	SS	F	P
Treatment	3	0.046	57.403	<0.001
Log Age	1	0.003	10.667	0.002
Pool	3	0.004	5.058	0.003
Residual	103	0.027		

Samples ranked by mean	MM	BM	MB	BB
Ranked least squares means	1.312	1.356	1.371	1.378
Size of samples	35	24	20	32

Comparison	Difference	SE	q	P
BB vs. MM	0.065	0.003	23.17	<0.001
BB vs. BM	0.021	0.003	6.81	<0.001
BB vs. MB	0.007	0.003	2.13	>0.20
MB vs. MM	0.058	0.003	18.06	<0.001
MB vs. BM	0.014	0.003	4.07	<0.05
BM vs. MM	0.044	0.003	14.45	<0.001

B. Standardized mass at metamorphosis

Source of variation	df	SS	F	P
Treatment	3	0.002	3.223	0.026
Log Age	1	0.001	4.932	0.029
Pool	3	0.003	4.223	0.007
Residual	103	0.025		

Samples ranked by mean	MM	BB	MB	BM
Ranked least square means	−0.003	−0.002	−0.002	0.009
Size of samples	35	32	20	24

Comparison	Difference	SE	q	P
BM vs. MM	0.012	0.003	4.08	<0.05
BM vs. BB	0.010	0.003	3.55	>0.05
MB vs. MM	0.001	0.003	0.486	>0.50

rearing conditions. Thus, *S. bombifrons* females can potentially produce faster-developing offspring by hybridizing than by mating with conspecifics.

By developing faster, BM tadpoles would be more likely than pure *S. bombifrons* tadpoles to escape a drying pond. Spadefoots breed in ephemeral pools, and their tadpoles often fail to metamorphose before their ponds dry (Pfennig 1992). Thus, in times and locations when ponds dry rapidly, *S. bombifrons* females may produce more surviving offspring by mating with a *S. multiplicata* male. However, to determine the precise circumstances under which hybridization might be beneficial for *S. bombifrons* females, we need to take into account the fact that hybrid males are sterile and female hybrids produce about half as many eggs as a pure *S. bombifrons* female (Simovich 1985; Simovich et al. 1991). Thus, to compensate for these fitness costs, *S. bombifrons* females must gain more than four times as many offspring by hybridizing than by mating with their own species (assuming a 1:1 sex ratio).

Is there any window of time during which more than four times as many BM tadpoles are metamorphosing than pure *S. bombifrons*? Between days 21 and 23 of the experiment reported here, BM hybrids were metamorphosing at a rate greater than five times that of BB offspring (Fig. 3). If the pools had dried during this period, BM hybrids potentially would have had sufficiently high survival in the larval period

to outweigh the known postmetamorphic costs. Similarly, previous work (Simovich 1985; Simovich et al. 1991) has shown that, when fed a natural diet of live fairy shrimp, BM larvae metamorphosed at rates greater than five times that of pure *S. bombifrons* over a five-day period. Thus, BM off-
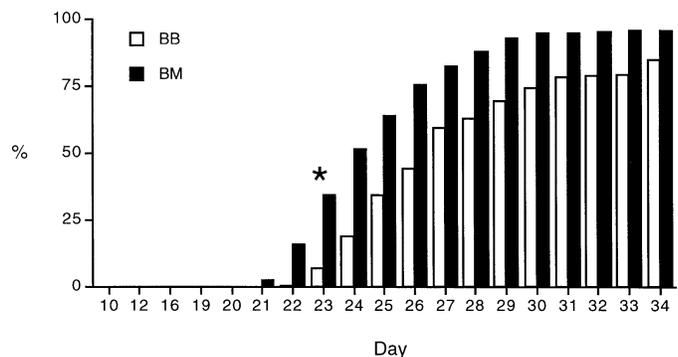

FIG. 3. Cumulative percent of metamorphs produced over the duration of the experiment compared between *Spea bombifrons × S. bombifrons* (BB) and *S. bombifrons × S. multiplicata* (BM) offspring. Asterisk indicates point at which BM offspring are metamorphosing at equal to or greater than five times that of BB.

spring can potentially have overall higher fitness than pure *S. bombifrons* offspring if they are more likely to escape a drying pool. Consequently, in highly ephemeral habitats, *S. bombifrons* females that hybridize may not necessarily be selected against.

Observations of natural ponds indicate that pond duration varies dramatically both spatially and temporally (K. Pfennig, unpubl. data; Pfennig 1992). Thus, the strength of selection against those *S. bombifrons* females that hybridize also will vary spatially and temporally. Overall, then, selection to avoid hybridization by *S. bombifrons* females will be weakened by the presence of environmental contexts in which hybrids can outperform parental types. Consequently, *S. bombifrons* females may not avoid heterospecifics as strongly as *S. multiplicata* females.

Field observations indicate that *S. bombifrons* females are indeed more likely to hybridize than *S. multiplicata* females (see Table 2, Fig. 1). The fact that mixed-species crosses are more likely to consist of *S. bombifrons* females than *S. multiplicata* females is striking, because spadefoot males aggregate in conspecific groups with *S. bombifrons* calling from the periphery and *S. multiplicata* calling from the center of the pond (Pfennig et al. 2000; see also Simovich 1985). Given this spatial distribution of males, a female *S. bombifrons* would therefore be less likely to encounter a heterospecific mistakenly than would a *S. multiplicata* female that must pass by heterospecifics to reach conspecific groups (cf. Lamb and Avise 1986). Perhaps more critically, F_1 hybrids are relatively more abundant in smaller, ephemeral ponds than in larger, long-lasting ponds (see Results), as is expected if hybrids are potentially favored in ephemeral habitats.

How *S. bombifrons* females come to hybridize more often than *S. multiplicata* females is unclear. *Spea bombifrons* females may facultatively adjust their mate preferences for conspecifics versus heterospecifics depending on the size of their breeding pond, or they may have less opportunity for mate choice in ephemeral pools, which can have higher densities of males that may intercept females (Simovich 1985; see also Wirtz 1999). Alternatively, female *S. bombifrons* may mate with heterospecifics if conspecific males are rare or difficult to locate (Wirtz 1999; Malmos et al. 2001). Behaviors that contribute to hybridization such as random mating, reduced vigilance against indiscriminately mating males, or mating with heterospecifics when conspecifics are rare may be evolutionarily maintained when selection against behaviors promoting hybridization is weak or nonexistent.

The discussion above focuses mainly on the fitness consequences of hybridization for females. Contrary to females that always mate successfully, males of both species may do better to hybridize if the alternative is not to mate. Thus, for males of each species, the fitness consequences of hybridization depend not only on the relative fitness of hybrid offspring versus pure-species offspring, but also on opportunities for mating with conspecific females. Interestingly, *S. multiplicata* males appear more likely than *S. bombifrons* males to engage in indiscriminate matings with heterotypic females (Table 2, Fig. 1). Moreover, *S. multiplicata* males are more likely to engage in active searching for mates than are *S. bombifrons* males (K. Pfennig, pers. obs.; M. A. Simovich, pers. obs.). One possible evolutionary explanation for this pattern is that the benefits of hybridizing are more likely to outweigh its costs for *S. multiplicata* males than for *S. bombifrons* males. Consequently, the possible benefits of hybridization for *S. multiplicata* males may contribute to introgression between the two species and the pattern of hybridization we observed.

Generally, the fitness consequences of hybridization may differ for the two interacting species, such that in extreme cases, one species may be selectively favored to avoid hybridization while the other species is selectively favored to engage in hybridization. Differential selection to avoid hybridization is especially likely when characters closely associated with fitness exhibit additive genetic variance (e.g., in the case of spadefoots, development time of the tadpoles was intermediate between the parental types).

Despite differential selection between the spadefoots to avoid hybridization, character displacement of both female preferences (Pfennig 2000) and male calls (Pierce 1976) indicate that reinforcement may be proceeding. Indeed, hybridization may be decreasing in the southeastern Arizona populations (K. Pfennig, unpubl. data). Such a finding suggests that the conditions under which reinforcement can occur may be less restrictive than previously thought (see also Kirkpatrick and Servedio 1999). Perhaps strong selection against hybridization by females of one species (*S. multiplicata*, in the case of the spadefoots) is sufficient to generate divergence in mating behaviors that promote further reproductive isolation.

Although reinforcement appears to occur in spadefoots, differential selection to avoid hybridization might impede reinforcement in other systems. Whether reinforcement proceeds is likely sensitive to both the strength of selection against hybridization and the amount of gene flow between populations (Liou and Price 1994; Kelly and Noor 1996; Servedio and Kirkpatrick 1997; Kirkpatrick and Servedio 1999). Thus, determining the conditions under which reinforcement can occur may require evaluating how selection to avoid hybridization varies between interacting species. Further theoretical and empirical work is required to assess how such selective dynamics result in reinforcement, adaptive introgression, or some balance between these two processes.

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