

REINFORCEMENT IN CHORUS FROGS: LIFETIME FITNESS ESTIMATES INCLUDING INTRINSIC NATURAL SELECTION AND SEXUAL SELECTION AGAINST HYBRIDS

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Maladaptive hybridization is hypothesized to be an important force driving the evolution of reproductive isolation between closely related species. Because the magnitude and direction of selection can vary across a life cycle, an accurate understanding of the ubiquity of reinforcement requires fitness to be estimated across the life cycle, but the literature is surprisingly depauperate of such studies. We present fitness estimates of laboratory-raised hybrids between the chorus frogs *Pseudacris feriarum* and *Pseudacris nigrita*—two species that have undergone reproductive character displacement where they come into secondary contact. By studying viability, mating success, and fertility across the life cycle, we find strong support for reinforcement as the force driving displacement in this system. Specifically, we find hybrid fitness is reduced by 44%. This reduction results from both sexual selection against hybrid males and natural selection on male fertility, but not viability selection. Sexual selection against hybrid males is four times stronger than natural selection. Hybrid female fitness is not reduced, however, suggesting that Haldane's rule may be operating in this system if males are heterogametic. We also found higher variation in hybrid male fertilization success relative to *P. feriarum* males, suggesting that the hybrid incompatibility genes are polymorphic within one or both of the parent species.

KEY WORDS: Hybrid incompatibility, hybrid inviability, hybrid sterility, hybridization, *Pseudacris*, reproductive character displacement.

Identifying the selective forces causing the evolution of reproductive isolation between populations is critical for understanding the origin and maintenance of biodiversity. Geographic regions of secondary contact between species can be particularly informative areas for investigating the evolution of reproductive isolation, and in some cases can provide windows into the process of speciation (Harrison 1990, 1993; Gröning and Hochkirch 2008; Ortiz-Barrientos et al. 2009; Pfennig and Pfennig 2009). In situations in which hybridization between incipient species is maladaptive,

selection may favor the divergence of species-recognition behaviors within the contact zone, resulting in a reduction of inter-specific matings through time and allowing the completion of reproductive isolation between diverging species (Liou and Price 1994; Kirkpatrick and Servedio 1999; Kirkpatrick 2000; Servedio 2000; Kirkpatrick 2001). This process, known as reinforcement (Dobzhansky 1937, 1940; Servedio and Noor 2003), has been documented in an increasing number of systems in recent years, including birds, mammals, insects, fish, frogs, and plants (Noor 1995; Saetre 1997; Rundle and Schluter 1998; Higgie et al. 2000; Pfennig and Simovich 2002; Pfennig 2003; Nosil et al. 2003;

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Ortiz-Barrientos et al. 2004; Hoskin et al. 2005; Peterson et al. 2005; Smadja and Ganem 2005; Jaenike et al. 2006; Kronforst et al. 2007; Nosil et al. 2007; Kay and Schemske 2008; Urbanelli and Porretta 2008).

The pattern of increased premating isolation in sympatry relative to allopatry that may result from reinforcement is known as reproductive character displacement (Brown and Wilson 1956; Howard 1993). To ascertain whether an apparent pattern of character displacement is due to reinforcement, Howard (1993) established five criteria: (1) heterospecific matings occur in nature, (2) hybridization is maladaptive, (3) the observed displacement is perceptible to the opposite sex, (4) variation is heritable, and (5) the displacement is not due to ecological or other factors. Of primary importance in this list is demonstrating evidence for selection against maladaptive hybridization. Theoretical and empirical work has shown that hybridization may be maladaptive as a result of intrinsic factors, such as hybrid inviability or hybrid infertility, or as a consequence of extrinsic factors, such as ecological inviability or behavioral (sexual) selection against hybrids (Servedio and Noor 2003; Coyne and Orr 2004). Studying all of these factors across the life cycle of an organism can be quite difficult and has only been done thoroughly in flycatcher birds (Saetre et al. 1997; Svedin et al. 2008). After almost three decades of work, a team of researchers has been able to estimate nearly all aspects of flycatcher hybrid fitness in nature, across multiple geographic regions (Alatalo et al. 1982, 1990; Saetre et al. 1997, 1999, 2002; Qvarnstrom 1999; Veen et al. 2001; Saetre 2002; Haavie et al. 2004; Borge et al. 2005; Qvarnstrom et al. 2006; Saether et al. 2007; Veen et al. 2007; Svedin et al. 2008; Veen et al. 2009; Wiley et al. 2009).

In most examples of reinforcement, however, researchers have focused on factors influencing hybrid fitness during a limited portion of the life cycle. Studying selection in this manner can be misleading because the magnitude and direction of selection can shift across the life cycle. For example, several studies have found that although hybrid fecundity is low, hybrid viability is equal or greater than parental crosses (Simovich 1985; Simovich et al. 1991; Parris 1999; Parris et al. 1999; Parris 2001a,b,c). A

series of pioneering studies in frogs obtained hybrid viability data for over 100 different species crosses (reviewed in Sasa et al. 1998, and Malone and Fontenot 2008). Although this body of data represents a tremendous resource, ~74% of these studies only estimated some aspect of larval viability (Table 1). Larval viability, however, may be a poor indicator of overall hybrid fitness because it may not correlate with postlarval fitness. To gain clearer insight into the cost of hybridization, fitness should be examined across the entire life cycle, to quantify viability and fertility of hybrids as well as their sexual attractiveness to potential mates.

The North American chorus frog genus *Pseudacris* (treefrog family Hylidae) is a promising system for investigating the evolution of reproductive isolation between species in the context of reinforcement. Two species, *P. feriarum* and *P. nigrita*, form a contact zone in the southeastern United States from Alabama to Virginia. The taxa diverged approximately eight million years ago and have since presumably come into secondary contact (Lemmon et al. 2007a,b). In both species, the majority of individuals are thought to breed only once, after reaching sexual maturity at one year (Caldwell 1987; E. M. Lemmon, unpubl. data). Within the contact zone, *P. feriarum* has undergone reproductive character displacement with respect to male acoustic signals and female preferences for these signals (Fig. 1; Fouquette 1975; Lemmon 2009). Male signals have displaced in different traits in different populations within sympatry (Lemmon 2009). Putative hybrids, which are acoustically and morphologically intermediate between the parental species, have been found in the contact zone (Lemmon 2009). Precise estimates of the frequency of hybridization from genetic data will be presented elsewhere (E. M. Lemmon, unpubl. data). Whereas the classic pattern expected from reinforcement (reproductive character displacement) and evidence of natural hybridization exist in this system, the evolutionary consequences of hybridization have not been studied. Here, we present a nearly complete picture of the fitness consequences of hybridization by *P. feriarum* females, including measures of lifetime intrinsic fitness (hybrid viability and sterility) as well as a measure of extrinsic fitness (sexual selection against hybrid signals).

Table 1. Life-history stages examined in studies of hybrid fitness in frogs. The majority of studies focused only on larval viability, and usually upon one small fitness component (e.g., hatching success). Note that most studies had very low sample sizes and many lacked control crosses (see Table S5 for an expanded version of this table and studies cited).

	Total	Larval viability	Postlarval viability	Mating success	F1 hybrid fertilization success	Lifetime fitness
Present study		Yes	Yes	Yes	Yes	Yes
Number of previous studies	38	32	0	5	6	0
Number of species crosses in previous studies	258	246	0	9	11	0

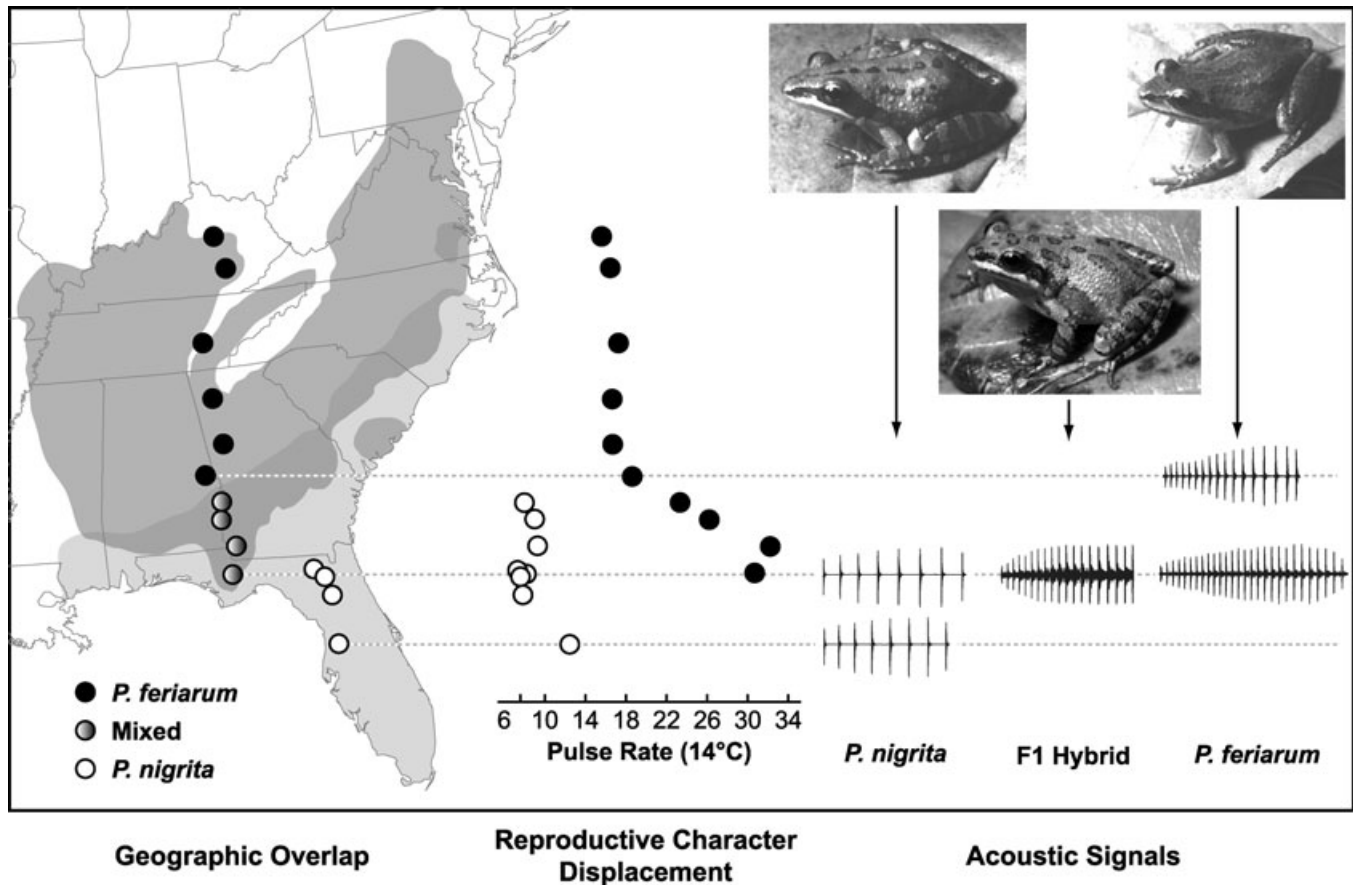


Figure 1. Reproductive character displacement results from geographic overlap between two species of chorus frog. Pulse rate of the advertisement call of *Pseudacris feriarum* is displaced in areas in which *P. nigrita* is present (adapted from Fouquette 1975). Oscillograms (amplitude—*x*-axis, plotted against time—*y*-axis) are shown for calls of allopatric and sympatric *P. feriarum*, allopatric and sympatric *P. nigrita*, and a laboratory-raised hybrid. All calls were recorded at $\sim 14^{\circ}\text{C}$ and are plotted on the same time scale. Note the increased pulse rate and pulse number of the sympatric *P. feriarum* call relative to the allopatric *P. feriarum* call. Also note that the hybrid call is intermediate with respect to the two sympatric calls.

To maintain tractability of this study, we focused solely on hybridization by female *P. feriarum* because only this species has undergone reproductive character displacement in the populations under study, and therefore, it is expected to experience a higher cost to hybridization (Lemmon 2009). We also quantified only hybrid male attractiveness to females for the sexual selection estimates because females are the choosy sex in this system and thus should easily obtain a mate regardless of their genotype. Finally, we focused on intrinsic viability factors and one measure of extrinsic (sexual) selection instead of ecologically driven extrinsic viability factors, due to the difficulty of estimating all variables simultaneously. Note that an accurate estimate of lifetime fitness would also include measures of ecological inviability, which we must reserve for future studies. For the sake of brevity, we refer to fitness estimates that combine components across the life cycle as “lifetime,” while acknowledging that measures of ecological viability selection would improve these estimates.

Methods

INTRINSIC HYBRID FITNESS: VIABILITY AND STERILITY

To study the influence of intrinsic factors on hybrid fitness, viability and sterility were assessed by comparing hybrid crosses to control parental crosses in the laboratory.

Hybrid viability

Amplexed conspecific pairs of *P. feriarum* and *P. nigrita* were collected from breeding sites in the Apalachicola National Forest (Liberty Co., Florida) on January 8, 2007. Pairs were separated, and each *P. feriarum* female was then paired with either a different *P. feriarum* male or a *P. nigrita* male to create 14 *P. feriarum* families and 15 hybrid families (Table S1). Each pair was placed in a plastic container with 1.5 L pond water and grass stems and allowed to mate naturally. After 12–24 h, adults were removed from the container and eggs were incubated at room temperature in the same container until hatching. We did not perform crosses using

P. nigrita females in this initial study (see Introduction and Discussion), because reproductive character displacement occurs only in *P. feriarum* in the populations studied here (Lemmon 2009).

After hatching, 30 tadpoles were haphazardly drawn from each family, divided into three groups, and placed into three new 1.5-L containers of dechlorinated neutral pH water (10 tadpoles in each). Thus each family was replicated three times. All containers were randomized spatially during the rearing process. Tadpoles were maintained on an ad libitum diet of chopped organic spinach with water changes approximately once per week. Each tadpole was removed from the container when it reached metamorphosis, which was defined as the first day in which both rear legs and at least one front leg had emerged. At this point, froglets were weighed, measured, and placed in a screened plastic tub containing damp sphagnum moss. Number surviving to metamorphosis and time to metamorphosis of individuals in each container were noted. Frogs were fed vitamin-dusted wingless fruitflies and crickets ad libitum until they reached sexual maturity at 10–12 months posthatching. Number surviving to sexual maturity in each family was noted. The tadpole-rearing component of the experiment was ended on May 7, at which point we assumed their natural ponds had dried.

Before proceeding with the statistical analyses, we determined whether the data could be pooled by family or by cross-type. To determine whether data from tubs within families could be pooled, we tested for significant among-tub variation for five measures for each family: larval survival, mass at metamorphosis, snout-vent length (SVL) at metamorphosis, time to metamorphosis, and survival to adulthood. Significance was assessed for each family using a randomization test in which the among-tub variance (for the measure of interest) was used as the test statistic. Each of the 10,000 samples from the null distribution was generated by computing the among-tub variance after tub identity was randomized across individuals within a family. A sequential Bonferroni test was used to correct for the 29 independent tests conducted for each measure (Rice 1989). Significance was assessed using a one-tailed test with $\alpha = 0.05$ because we were interested in whether there was greater variation across tubs than expected by chance.

We also determined whether data from different families within each cross-type could be pooled using an approach similar to that described above for among-tub variation. A test was conducted for each cross-type and for each of the five measures listed above. The variance across families within a cross-type served as the test statistic. Each of the 10,000 samples from the null distribution was generated by computing the among-family variance after family identity was randomized across individuals within a cross-type. Significance was assessed using a one-tailed test with $\alpha = 0.05$ because we were interested in whether there was greater variation across families than expected by chance.

We calculated the strength of viability selection against hybrids as $S_V = z(1 - \min(V_p, V_h)/\max(V_p, V_h))$, where $z = -1$ if $V_p > V_h$ and $z = 1$ otherwise, and V_p and V_h are the proportions of hatchlings that survived to adulthood, averaged across *P. feriarum* and hybrid families, respectively. Note that when selection coefficients are calculated in this manner, selection against hybrids results in a negative selection coefficient. Significance was assessed using a two-tailed randomization test in which S_V served as the test statistic ($\alpha = 0.05$). Each of the 100,000 samples from the null distribution was generated by recomputing S_V after randomizing the assignment of cross-type (hybrid or *P. feriarum*) to family.

Hybrid sterility

To test for the presence of male or female hybrid sterility, second-generation crosses were created using laboratory-raised *P. feriarum* and hybrid *P. feriarum* \times *P. nigrita*. These species and their hybrids reach sexual maturity at 10–12 months posthatching. We used a split-clutch and split-sperm design, such that each replicate consisted of four individuals (a female *P. feriarum*, a female F1 hybrid, a male *P. feriarum*, and a male F1 hybrid). In a split-clutch design, a female's eggs are divided and then fertilized by sperm from different males. In a split-sperm design, a male's testes are divided and used to fertilize eggs from multiple females. We did not include individuals from the same first-generation family within a replicate. This design allowed us to control for variation across individuals within a cross-type. Using in vitro fertilization, we mated each male to two females and each female to two males. The resulting crosses were: (1) *P. feriarum*, (2) hybrid female backcross, (3) hybrid male backcross, and (4) F2 hybrid (Table S2). To bring the frogs into breeding condition, adults were injected 8–12 h prior to the experiment with 500 IU (females) or 200 IU (males) of human chorionic gonadotropin (HCG) hormone (Lynch et al. 2006). When females had ovulated, the two males within a replicate were quickly dissected and each testis was macerated in one of four prepared petri dishes of Holtfretter's solution. Approximately 50 eggs were gently expressed from females in an alternating fashion into the dishes (two dishes per female). Approximately 24 h later, fertilization success (proportion of eggs fertilized) was quantified under a dissecting microscope by verifying cell division. The experiment was ended at this stage. Of the 29 original families created for the viability portion of this study, 23 were represented in the second-generation crosses (13 of 15 F1 hybrid families and 10 of 14 *P. feriarum* families). In these crosses, male *P. feriarum* were taken from eight of the 14 families, male F1 hybrids from 11 of the 15 families, female *P. feriarum* from 11 of the 14 families, and female F1 hybrids from six of the 15 families (Table S2).

The strength of fertility selection (S_F) was calculated based on fertilization success for F1 individuals backcrossed to *P. feriarum* relative to pure *P. feriarum* crosses. We assumed,

therefore, that formation of F2 hybrids and *P. nigrita* backcross hybrids occurs only rarely in nature (see Discussion). Fertilization success was also computed separately for males and females. In each case $S_F = (1/N) \times \text{SUM}(z_i(1 - \min(F_{pi}, F_{hi})/\max(F_{pi}, F_{hi})))$, where F_{pi} and F_{hi} are the proportions of eggs fertilized by the *P. feriarum* and hybrid individuals in cross i , $z_i = -1$ if $F_{pi} > F_{hi}$ and $z_i = 1$ otherwise. The sum is taken over all N crosses in which eggs could be obtained from the females (some females produced no eggs). Significance was evaluated using a two-tailed randomization test in which S_F was the test statistic ($\alpha = 0.05$). Each of the 100,000 samples from the null distribution was generated by recomputing S_F after randomizing the assignment of parents (*P. feriarum* or hybrid) to eggs within each cross.

Variation in hybrid sterility

Recent studies have demonstrated that hybrid incompatibility genes can be polymorphic within species (Good et al. 2007; Lopez-Fernandez and Bolnick 2007), which contrasts with most theoretical models of speciation that assume fixed differences between species at these loci. To determine whether this phenomenon may be occurring in chorus frogs, we measured variation in sterility within each class of hybrids and *P. feriarum* individuals. More specifically, we modeled fertilization success of a cross as an interaction between the fertilization potentials of the two individuals involved in the cross, such that fertilization success = male fertilization potential \times female fertilization potential. Note that female fertilization potential is the expected proportion of eggs that would be fertilized by a perfectly fertile male and vice versa. To model variation across individuals, we assume that fertilization potentials for individuals in each class (*P. feriarum* females, F1 hybrid females, *P. feriarum* males, and F1 hybrid males) are beta-distributed, with lower bound equal to 0 and upper bound equal to 1. The shapes of the four distributions, each described by the two parameters α and β , are the foci of the estimation. We further assume that when two individuals are crossed, the resulting number of fertilized eggs is described by a binomial distribution in which the probability parameter is simply the product of the fertilization potentials of the two individuals involved in the cross. The mathematical details of this model are given in the Supporting Information.

We used this statistical model of fertilization and a Markov chain Monte Carlo (MCMC) approach (Metropolis et al. 1953; Hastings 1970) to estimate the Bayesian posterior distribution of parameters describing the fertilization potentials for the four classes. Uniform priors (bounded between 0 and 200) were assumed for all eight parameters of the model. Sixteen independent Bayesian runs were performed to assess convergence and mixing of the Markov chains. Each Markov chain was run for 350,000 generations and sampled every 100 generations. The posterior distribution was estimated from samples obtained after stationarity

and convergence were reached. From the posterior distribution, we computed the mean and standard deviation in fertilization potential for each of the four classes. See Supporting Information for additional details of this analysis.

EXTRINSIC HYBRID FITNESS: SEXUAL SELECTION AGAINST HYBRID ACOUSTIC SIGNALS

To assess the strength of sexual selection against hybridization, hybrid male signals were recorded from laboratory-raised and wild frogs, and female preferences for hybrid signals compared to *P. feriarum* signals were quantified through three types of binary choice tests.

After the HCG injections above, but prior to dissection, signals of laboratory-raised male frogs were recorded. Ten males were placed in each of two screened-lid kiddie pools (by cross-type) containing pots of grass and 6 inches of water. Breeding conditions were simulated by raining on the pools for 4–6 h and cycling water via sprinkler heads above the pools while a *Pseudacris* chorus was played in the background. After this period, the sprinklers were turned off, and a single individual with a lower amplitude background chorus was played, to encourage duetting. Male signals were recorded at 44,100 Hz sampling rate using a Sennheiser (Lyme, CT) ME67 directional microphone and a Marantz (Mahwah, NJ) PMD660 digital recorder. Substrate temperature at the calling site was noted. A total of seven hybrid and five *P. feriarum* laboratory-raised frogs were successfully recorded (Table S1). Hybrid males were recorded at temperatures ranging from 14.0°C to 15.6°C whereas *P. feriarum* males were recorded at temperatures ranging from 14.6°C to 17.0°C.

Signals were analyzed using SoundRuler version 0.941 (<http://soundruler.sourceforge.net/>). Frequency measurements were taken from spectrograms generated with fast Fourier transform (FFT) length of 1024 and 900 samples of overlap among subsequent FFTs. Signal variables were either taken directly from SoundRuler's raw data output or calculated from these data. Pulse rate was temperature-corrected to 18°C for the preference tests based on a previously published relationship for *P. feriarum* (Lemmon 2009) and a similar relationship for hybrids that was determined by recording a single hybrid individual at 14, 14.2, 15, and 15.6°C. Stimuli for the preference tests were synthesized from 13 signal variables using the program JOSHSYN (written by Joshua Schwartz). Synthetic rather than natural calls were used to remove background noise of other individuals in the breeding chorus. Details of the acoustic analyses and stimuli synthesis are described in Lemmon (2009).

Binary preference tests were performed on wild *P. feriarum* females from a Liberty Co., Florida population on 15 February 2009 (Table S3). Females were given a choice between a hybrid and a *P. feriarum* call in three different experiments. Three sets of stimuli were presented to test repeatability of choices and to

ensure that any apparent preference was not merely a function of the specific pair of stimuli presented. The experiments were as follows: (A) randomly-drawn hybrid versus randomly-drawn *P. feriarum* (both laboratory-raised frogs, from a pool of seven hybrids and five *P. feriarum*); (B) average hybrid versus average *P. feriarum* (calculated from pool of laboratory-raised frogs); (C) one of two putative hybrids versus average *P. feriarum* (all wild-caught; Lemmon 2009). In experiment A, stimuli were drawn randomly for each female. Females were allowed 15 min to choose a stimulus; if no choice was made during this period, the test was terminated. Preference tests were conducted following the procedures of Lemmon (2009).

To assess the null hypothesis of no preference (proportion choosing each stimulus = 0.5), one-tailed exact binomial tests were conducted for each of the three experiments. One-tailed tests were used because the a priori expectation was that wild *P. feriarum* females would prefer the conspecific signal over the hybrid signal. A sequential Bonferroni correction was applied to account for multiple (3) tests (Rice 1989).

The strength of mating selection (S_M) was calculated based on ability of hybrid and *P. feriarum* males to attract females during the phonotaxis experiment. More specifically, we calculated the strength of mating selection as $S_M = z(1 - \min(M_p, M_h)/\max(M_p, M_h))$, where M_p and M_h are the numbers of females that chose the *P. feriarum* and hybrid calls, respectively, and $z = -1$ if $M_p > M_h$ and $z = 1$ otherwise. Significance was evaluated using a two-tailed randomization test in which S_M was the test statistic ($\alpha = 0.05$). Each of the 100,000 samples from the null distribution was generated by randomizing the assignment of cross-type to the call for each phonotaxis test.

LIFETIME FITNESS OF HYBRIDS

Lifetime selection against hybrids was computed for males, females, and all individuals as $S_L = z(1 - \min(W_h, W_p)/\max(W_h, W_p))$, where W_h is the lifetime fitness of the hybrids, W_p is the lifetime fitness of the *P. feriarum* individuals, $z = -1$ if $W_p > W_h$, and $z = 1$ otherwise. The lifetime fitness measures were calculated as $W_h = V_h \times M_h \times F_h$ and $W_p = V_p \times M_p \times F_p$. Because fecundity data were sampled in a paired fashion, F_h and F_p could not be computed directly, but instead were calculated from the selection coefficient as $F_h = 1 + S_F$ and $F_p = 1$ if $S_F < 0$, and $F_h = 1$ and $F_p = 1 + S_F$ otherwise. Significance was computed using a two-tailed randomization test in which S_L was the test statistic ($\alpha = 0.05$). Each of the 100,000 samples from the null distribution was generated by randomizing each of the three components as described above and recomputing S_L .

ECOLOGICALLY RELEVANT VIABILITY FACTORS

Several measures that may correlate with ecological factors are not captured in our lifetime fitness estimates. Those measures are:

body size at metamorphosis (SVL), mass at metamorphosis, and time to metamorphosis. Studies of other amphibian species have shown that larger and/or earlier-metamorphosing tadpoles have higher adult fitness (e.g., Altwegg and Reyer 2003). We conducted randomization tests for these measures to determine whether hybrids and *P. feriarum* individuals differed significantly. For each randomization test, we computed the mean value of the measure within families, computed the mean value across families (within each cross type), then computed the difference between the hybrid and *P. feriarum* means (hybrid mean – *P. feriarum* mean). Each of the 10,000 samples from the null distribution was generated by recomputing this quantity after cross-type identity (hybrid or *P. feriarum*) was randomized across families. Significance was assessed using a two-tailed test with $\alpha = 0.05$.

Results

DATA POOLING

No significant among-tub variation was found for all families and measures (Table S4). Consequently, all data were pooled within families for subsequent tests. Significant among-family variation was found in F1 hybrids for larval survival ($P < 0.0001$), time to metamorphosis ($P = 0.0068$), mass at metamorphosis ($P = 0.0040$), and survival to adulthood ($P < 0.0001$). Significant among-family variation was found in *P. feriarum* for larval survival ($P = 0.0001$), SVL at metamorphosis ($P = 0.0224$), and survival to adulthood ($P = 0.0014$). As a result, we did not pool families within cross-types for any of the tests below.

INTRINSIC HYBRID FITNESS: VIABILITY AND STERILITY

Hybrid viability

No significant difference was found between the viability of F1 hybrids and *P. feriarum* (Fig. 2; $S_V = 0.14184$, $P = 0.19131$). Similar results were found when just males ($S_V = 0.26984$, $P = 0.10930$) or just females ($S_V = 0.04016$, $P = 0.45235$) were considered. For these tests, we assumed that the sex ratio at hatching was 1:1. Observed sex ratios in adult frogs were 51.93% males in hybrids and 42.72% males in *P. feriarum*.

Hybrid sterility

F1 hybrid males had significantly lower fertilization success than *P. feriarum* males, after controlling for variation among females through the split-clutch design (Figs. 2 and 3; $S_F = -0.23016$, $P < 0.00001$). In contrast, F1 hybrid females had significantly higher fertilization success than *P. feriarum* females, after controlling for variation among males through the split-sperm design (Figs. 2 and 3; $S_F = 0.07016$, $P = 0.00087$). When male and female results were combined (assuming an equal secondary sex ratio, where male and female selection coefficients were given equal weight),

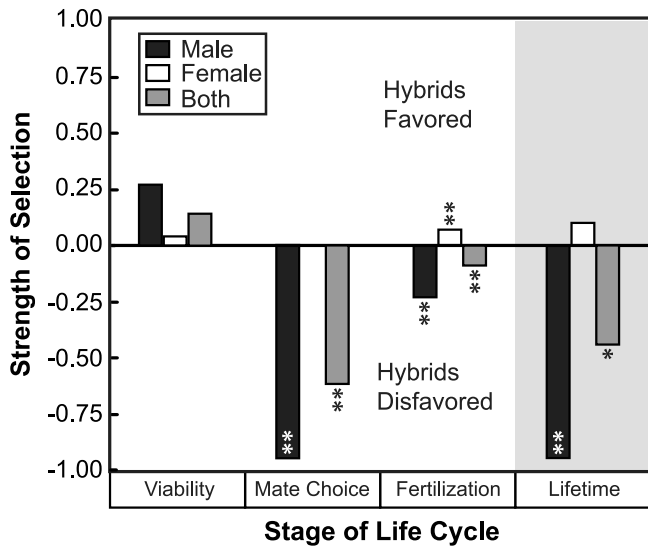


Figure 2. Variation in hybrid fitness across the life cycle. Bars above zero indicate that hybrids are favored relative to *P. feriarum* individuals, whereas bars below zero indicate that hybrids are disfavored. Strengths of selection on males, females, and combined individuals are shown (proportional to bar length). Single asterisk (*) is used to denote a *P*-value between 0.025 and 0.01, and two asterisks (**) are used to denote a *P*-value less than 0.01. Specific selection coefficients and *P*-values are shown in text. Finally, the gray panel on right highlights results for the overall strength of selection across the life cycle.

F1 hybrids overall had significantly lower fertilization success than *P. feriarum* (Fig. 2; $S_F = -0.08816$, $P < 0.00001$).

For the Bayesian model of fertilization potential, all 16 MCMC runs converged on the same distribution of parameter values by sample 600 of 3500. Consequently, we chose to estimate the posterior distribution by pooling the last 2500 samples collected during each run (40,000 samples total).

Results from the Bayesian model suggest that hybrid males have significantly higher variation in fertilization potential than any other class of individuals (posterior probability (PP) = 1.0; Table 2; Fig. 4). In fact, the standard deviation in fertilization potential was estimated to be almost two times that of *P. feriarum* males and nearly 10 times that of either female class. Moreover, the distribution of fertilization potentials for hybrid males was found to be significantly bimodal (PP($\alpha < 1$ and $\beta < 1$) = 1.0). This bimodal shape contrasts with the significantly unimodal shapes of the fertilization potential distributions for *P. feriarum* males, *P. feriarum* females, and hybrid females (Table 2).

EXTRINSIC HYBRID FITNESS: SEXUAL SELECTION AGAINST ACOUSTIC SIGNALS

Hybrid male signals were intermediate between the parental species *P. feriarum* and *P. nigrata* with respect to the two signal characters that have undergone reproductive character displace-

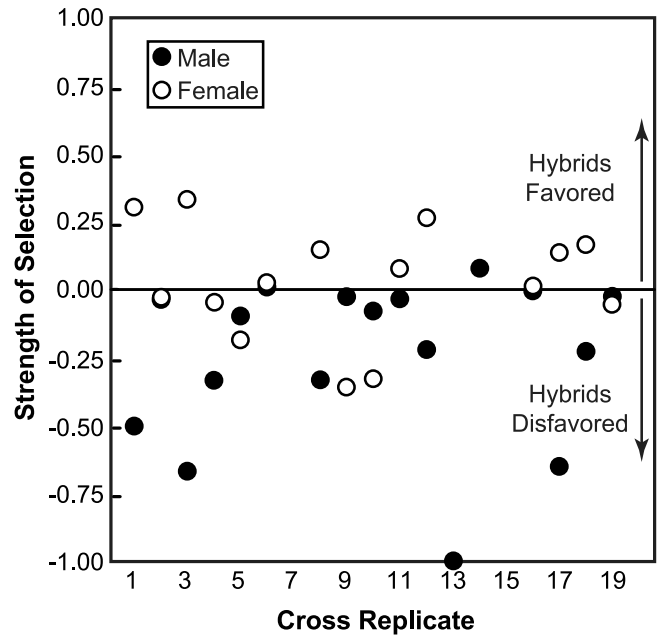


Figure 3. Estimates of fertilization success for F1 hybrid backcrosses relative to *P. feriarum* crosses. Each four-way cross consists of two hybrids and two *P. feriarum* in a split-clutch and split-sperm design. Selection coefficients, which were calculated as described in the Methods, are positive when the hybrid has higher fertilization success than the *P. feriarum* individual, and negative when the reverse is true. Split-clutch results revealing strong fertility selection against male hybrids are indicated by closed circles. Split-sperm results revealing weak fertility selection against *P. feriarum* females are indicated by open circles. Note that some points are missing because some females failed to produce eggs (Table S2).

ment in sympatry (pulse rate and pulse number; Table 3). Signals of laboratory-raised hybrids overlapped in these characters with wild hybrids, and signals of laboratory-raised *P. feriarum* overlapped with those of wild *P. feriarum*, suggesting that pulse rate and pulse number are heritable characters (Fig. 5; Table 3).

In the preference tests, female *P. feriarum* strongly preferred the signals of conspecific males over F1 hybrid males in all three tests (Test A: 20 of 20 females, $P < 0.00001$; Test B: 20 of 20 females, $P < 0.00001$; Test C: 19 of 20 females, $P < 0.00001$). All 20 females tested made a choice in all three tests.

The randomization tests indicated that F1 hybrid males were significantly less attractive to females than *P. feriarum* males ($S_M = -0.94717$, $P < 0.00001$). To be conservative, results from Test C were used in this calculation. Note that this selection coefficient is approximately four times greater than the male sterility selection coefficient. To calculate a combined sexual attractiveness selection coefficient for males and females, we assumed, based on observation of wild frogs, that hybrid females could obtain mates as easily as *P. feriarum* females ($S_M = 0$). Under this assumption, and the observed sex ratios in laboratory-raised adult

Table 2. Estimates of the distributions of fertilization potential for *P. feriarum* males (M_p), hybrid males (M_h), *P. feriarum* females (F_p), and hybrid females (F_h). Mean values (across samples from the Bayesian posterior distribution) of α and β , the two parameters of the beta distribution used to model the fertilization potential in each class, are given. Posterior probabilities (PP) of the distributions being unimodal or bimodal are also given. Means (μ) and standard deviation (σ) of each distribution was computed from mean estimates of α and β .

	α	β	PP (unimodal)	PP (bimodal)	μ	σ
M_p	1.884971	0.3554023	0.9924	0.0076	0.8419861	0.2033371
M_h	0.4778479	0.3581351	0	1	0.5736126	0.3643425
F_p	136.1662	2.589952	1	0	0.9809345	0.0120956
F_h	128.9720	1.276328	1	0	0.9896899	0.0093578

frogs (see above), we found that F1 hybrids overall experienced strong negative sexual selection ($S_M = -0.6150$, $P = 0.00003$).

CUMULATIVE LIFETIME FITNESS

Hybrid males had significantly lower lifetime fitness than *P. feriarum* males (Fig. 2; $S_L = -0.94416$, $P < 0.00001$). Hybrid females did not have significantly different lifetime fitness than *P. feriarum* females (Fig. 2; $S_L = 0.10016$, $P = 0.26200$). When males and females were combined, however, hybrids overall had significantly lower lifetime fitness than *P. feriarum* (Fig. 2; $S_L = -0.44017$, $P = 0.01510$). The lifetime fitness of hybrids relative to *P. feriarum*, therefore, was estimated to be $W_h/W_p = 0.55983$.

ECOLOGICALLY RELEVANT FACTORS

For body size, no significant difference was found between hybrid and *P. feriarum* individuals at metamorphosis (test stat. = -0.0057 cm, $P = 0.6883$) and at adult stages (Fig. 6; test stat. = -0.05204 cm, $P = 0.8613$). No significant difference was found between hybrid and *P. feriarum* individuals for mass at metamorphosis (test stat. = 0.002849 g, $P = 0.1627$) or time to metamorphosis (test stat. = 5.820717 days, $P = 0.0257$). Field experiments in natural ponds are required to evaluate how hybrid fitness may be reduced by a ~ 6 -day delay in time to metamorphosis. Note that hydroperiod is expected to vary substantially across ponds and across years.

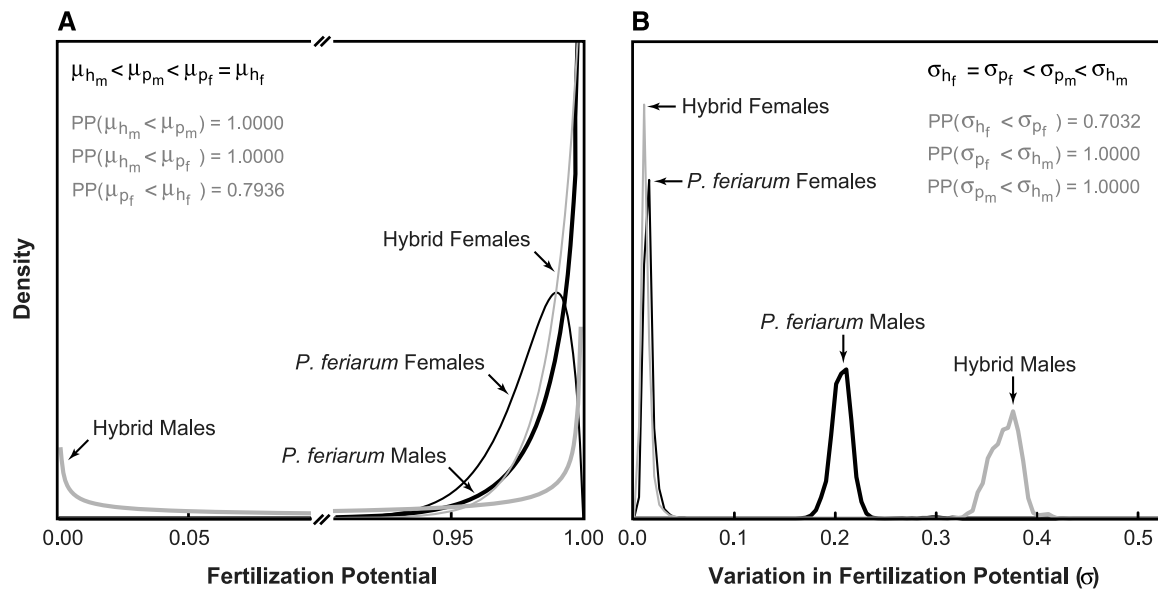


Figure 4. Fertilization potential for hybrid and *P. feriarum* males and females. Each curve in panel A represents an estimated distribution of fertilization potentials (based on the mean estimates for the beta parameters). Each curve in panel B represents the corresponding Bayesian posterior probability distribution of the standard deviation in the fertilization potentials (computed from the estimated beta parameters, see Supporting Information for details). We also present results from Tukey-type multiple comparison tests in which we used posterior probability (PP) estimates to test for significance among mean fertilization potential (μ ; panel A) and standard deviation in fertilization potential (σ ; panel B). Note that the distribution of hybrid male fertilization potential is bimodal and that the standard deviation in fertilization potential of hybrid males is nearly two times that of *P. feriarum* males. No significant difference was observed between the variances in fertilization potentials of the two female types.

Table 3. Variation in male acoustic signals among wild and laboratory-raised *P. feriarum*, *P. nigrata*, and F1 hybrids. Mean (bold), standard deviation, and range of values are shown. Definitions of signal components are given in Lemmon (2009). Note that hybrid call characters are intermediate between the parental species. Also note that signals of laboratory-raised individuals overlap with those of wild individuals within each class, suggesting that these characters are heritable (Fig. 5).

	<i>N</i>	Pulse rate	SD	Range	Pulse number	SD	Range
Laboratory <i>P. feriarum</i>	5	33.75	2.84	30.39–36.61	29.13	3.59	23.70–32.71
Wild <i>P. feriarum</i>	20	30.68	2.46	26.92–35.84	24.33	3.60	19.13–31.00
Laboratory hybrids	7	20.26	1.70	17.31–22.69	15.69	2.22	13.38–19.86
Wild hybrids	2	16.94	2.87	14.91–18.97	14.85	0.98	14.16–15.54
Wild <i>P. nigrata</i>	20	8.54	1.10	6.47–10.62	9.65	1.47	7.24–11.68

Discussion

Our study provides the first conclusive evidence for reinforcement driving the evolution of reproductive isolation between *P. feriarum* and *P. nigrata* and represents one of the strongest cases for reinforcement in frogs. Hybrids suffer a 44% reduction in fitness relative to *P. feriarum* across the life cycle. The strongest postzygotic isolating mechanisms between the two species are sexual selection against male hybrid mating signals ($S_M = -0.95$) and partial hybrid male sterility ($S_F = -0.23$). One striking finding is the fact that sexual selection against male hybrids is four

times stronger than natural selection (assuming male sterility is not attributable to postmating prezygotic sexual selection). These forces may be weakly moderated by marginally higher lifetime viability in hybrids. We also found that hybrid male fertility is bimodally distributed, suggesting within-species polymorphism of hybrid sterility genes. Here, we have shown evidence for selection against hybridization, the foremost of Howard's (1993) criteria for demonstrating that reproductive character displacement is due to reinforcement. Howard's (1993) other four criteria are also met: (1) hybridization occurs in nature (Lemmon 2009; E. M. Lemmon, unpubl. data), (2) displacement of the male signal is perceptible to the opposite sex (Lemmon 2009), (3) variation is heritable (Table 3; Fig. 5; E. M. Lemmon, unpubl. data), and (4) displacement of signals and preferences has not occurred for ecological reasons only (Lemmon 2009). This last criterion will be tested more thoroughly in future work.

A weakness of many previous studies examining the potential for reinforcement is that hybrid fitness is only studied during a small portion of the life cycle. In frogs, for example, the great majority of studies have only examined some aspect of larval viability (e.g., fertilization success, hatching success, etc.), ignoring potentially important factors such as postlarval viability, hybrid sterility, and sexual selection against hybrids (Table 1; Table S5). Moreover, no other frog studies have attempted to estimate overall lifetime fitness of hybrids. This tendency could lead to a biased estimate of the ubiquity (or lack thereof) of reinforcement across taxa. If larval viability is not a good predictor of lifetime fitness, as found in this study, we expect that the extent of reinforcement has probably been underestimated in this clade. The primary reason for the dearth of information on other aspects of hybrid fitness is the difficulty of maintaining animals in the laboratory to sexual maturity. Whereas larval viability data can be obtained in a matter of days or weeks, collecting data from the later life-history stages generally requires at least 1–2 years. Unfortunately, no method exists to short-cut this process (but see Lemmon 2007 and Wiley et al. 2009).

Although our study represents a single datapoint (species pair), the results are consistent with previous work suggesting

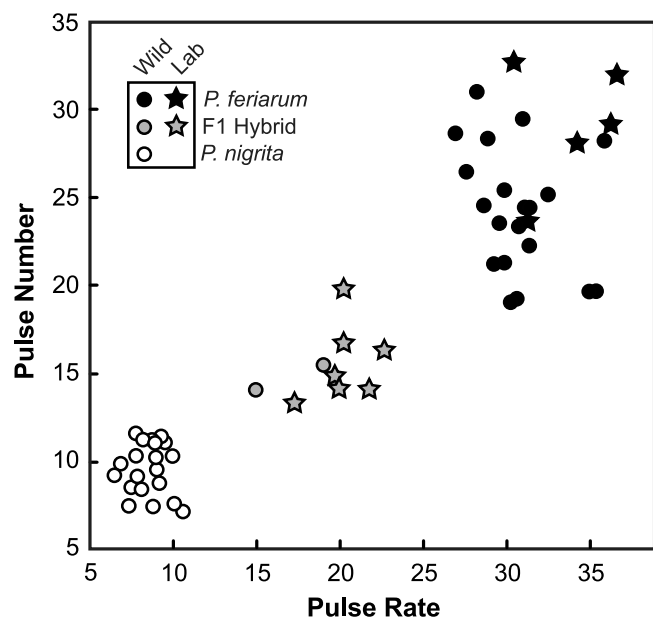


Figure 5. Characteristics of acoustic signals of wild-caught and laboratory-raised *P. feriarum*, F1 hybrids, and *P. nigrata*. The two call characters (pulse rate and pulse number) that displaced in sympatry are plotted. Note that hybrid signals are intermediate in both characters between the parental species. Note also that for both hybrids and *P. feriarum*, signals of laboratory-raised individuals overlapped with those of wild-caught frogs for each character. Signals of wild-caught frogs were taken from Lemmon (2009). For consistency with Lemmon (2009), pulse rate was corrected to 14°C in this figure (see Methods).

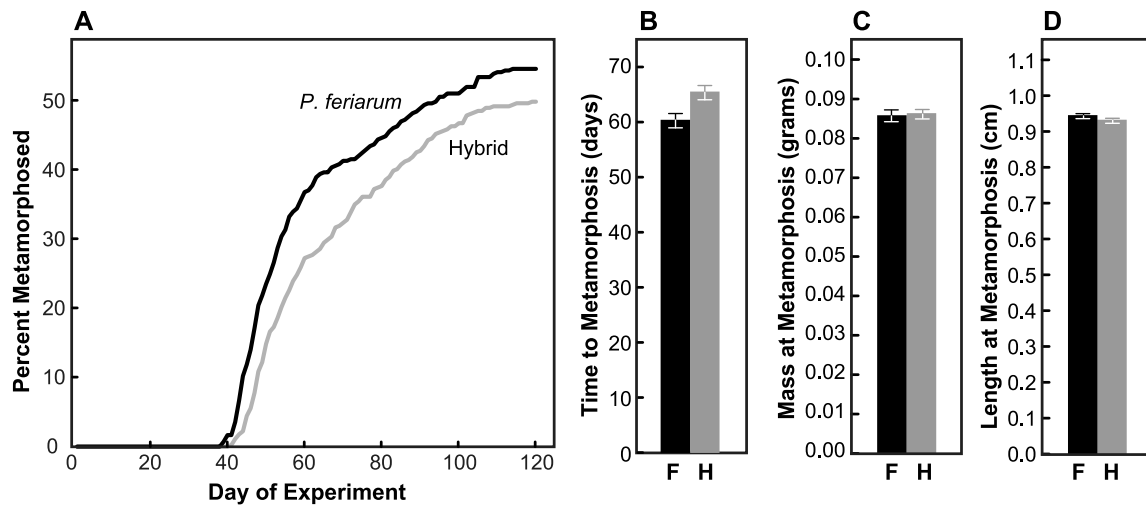


Figure 6. Ecologically relevant measures of larval fitness for hybrids (H) and *P. feriarum* (F) individuals. Four measures are shown: (A) percent metamorphosed over course of experiment, (B) average time to metamorphosis for surviving individuals, (C) mass at metamorphosis, and (D) snout-vent length at metamorphosis. None of these four measures were significantly different between hybrids and *P. feriarum* (see Results). Note, however, that these measures are connected to ecology via hydroperiod, one factor that was not manipulated in this study. Error bars indicate standard error in mean estimates.

that hybrid sterility evolves before hybrid inviability (Coyne and Orr 1989, 1997; Sasa et al. 1998; Presgraves 2002). Furthermore, our data are consistent with the finding that strong mating discrimination evolves before severe hybrid sterility or inviability (for sympatric taxa: Coyne and Orr 1989; and for allopatric taxa: Mendelson 2003). Whereas we cannot ascertain the order of evolution of these isolating mechanisms, our work suggests that mating discrimination is nearly complete (Fig. 2) but hybrid sterility genes are not yet fixed for alternative alleles in the two species (Figs. 3 and 4). Although male heterogamety has not been established in *Pseudacris*, most other members of the treefrog family Hylidae that have been studied do suggest this pattern (Kawamura and Nishioka 1977; Anderson 1991; Schmid and Steinlein 2003; Wiley 2003; Berset-Brändli et al. 2006; but see *Hyla squirella*, Anderson 1991). If *P. feriarum* and *P. nigrita* males are indeed heterogametic, the results of our study would be consistent with Haldane's rule (Haldane 1922; Orr 1997) because male hybrids show partial sterility, whereas hybrid females do not.

Intraspecific polymorphism in hybrid incompatibility genes, specifically those involved in hybrid sterility, is suggested by the fact that male hybrids showed substantially higher variation in fertilization success than *P. feriarum* males or females of either cross-type (Fig. 4B). Further, we demonstrated that the fertilization potential of male hybrids is bimodally distributed (Fig. 4A), indicating that some males had high fertilization success whereas others had very low success. This pattern is consistent with recent studies demonstrating intraspecific variation in hybrid inviability genes (Lopez-Fernandez and Bolnick 2007) and hybrid sterility genes (Good et al. 2007) in fish and mice, respectively. In contrast

to the fertilization data, acoustic signals of hybrid males were all intermediate between the two parental species (Table 3; Fig. 5). This finding suggests instead that either the genes controlling signal characters are not polymorphic within species or many genes control this complex trait. Currently, however, the genetic architecture underlying the frog acoustic signal is not well understood.

One unexpected result from our study of sterility in second-generation crosses was that hybrid females had a small but significant advantage over pure females (Figs. 2 and 3). One possible explanation for this pattern is slightly different responses to injection of hormones for *P. feriarum* and hybrid females (i.e., the time between injection and peak egg maturity differs between cross-types). The small advantage of hybrid females, however, is more than offset by the substantial disadvantage of hybrid males (Fig. 2).

We found that sexual selection against hybrids was the most important factor contributing to postzygotic isolation between *P. feriarum* and *P. nigrita* (Fig. 2). Hybrid male signals are intermediate in structure between the parental species, and are strongly rejected by females (Fig. 5). Whereas studies of most empirical systems are still too incomplete to ascertain its relative importance, sexual selection against hybrids has been demonstrated in diverse taxonomic groups, including butterflies (Naisbit et al. 2001), grasshoppers (Bridle et al. 2006), walking stick insects (Nosil et al. 2003, 2007), spiders (Stratton and Uetz 1986), flycatchers (Svedin et al. 2008), sticklebacks (Vamosi and Schluter 1999), and treefrogs (Höbel and Gerhardt 2003). These patterns are supported by theoretical work suggesting that sexual selection against hybrids can contribute to reinforcement under some

conditions (Coyne and Orr 1998; Kirkpatrick and Servedio 1999; Servedio 2004). In a study of the basis for reinforcement in flycatchers, Svedin et al. (2008) attempted to separate the effects of natural and sexual selection against F1 hybrids and found that sexual selection is the strongest component of hybrid unfitness. Our results are consistent with this finding.

Despite the fact that our study captures several very important features of lifetime hybrid fitness, several aspects could be improved and expanded in future work. One way to increase the power of our F1 viability experiments, for example, would be to use a split-clutch design (e.g., Pfennig 2000), similar to the design we used for the fertility experiments. Although we do not expect this design to affect the estimated viability or lifetime selection coefficients obtained in this study, it may change the significance level of the viability tests. Second, in this study we focused on the fitness consequences of *P. feriarum* females choosing heterospecific males over conspecific males in the breeding chorus. Our rationale for targeting this direction of hybrid cross is that *P. feriarum* is the species that has undergone reproductive character displacement and therefore is expected to suffer a greater fitness reduction due to hybridization (Fouquette 1975). An interesting future direction would be to investigate the fitness consequences of the opposite direction of hybrid cross. Given the lack of character displacement in signals of *P. nigrita*, and the apparently widespread phenomenon of asymmetry in hybrid fitness across taxa (Bolnick et al. 2008), we might expect the fitness costs of hybridization by *P. nigrita* females to be lower than in *P. feriarum*. Third, in the present study we focused on one direction of F1 hybrid backcross (to *P. feriarum* only), and on the attractiveness of hybrid male signals to female *P. feriarum* only. Clearly, the work we present here should be expanded to examine the effects of reproductive interactions with *P. nigrita* as well. Finally, we did not include ecological factors (e.g., Hatfield and Schluter 1999; Rundle 2002; Pfennig et al. 2007) in our laboratory study, such as natural diet, and therefore this work is primarily restricted to the effect of intrinsic factors on hybrid fitness (excepting sexual selection). We did measure several ecologically relevant life-history traits (i.e., time to, body length at, mass at, and size at metamorphosis), but found no significant differences in these measures between hybrid and *P. feriarum* individuals (Fig. 6). These traits are connected to viability measures because in nature, the faster growing and faster metamorphosing tadpoles are able to escape drying ponds or increasing densities of aquatic predators (Pfennig 2007). Future work will estimate hybrid fitness in an ecological context.

CONCLUSION

This study demonstrates that reinforcement has driven reproductive character displacement of female preferences and male signals between *P. feriarum* and *P. nigrita*. Moreover, our work indi-

cates that the direction and magnitude of selection against hybrids varies by sex and life-history stage, underscoring the importance of studying hybrid fitness across the entire life cycle. Stronger selection against hybrid males than hybrid females suggests that Haldane's rule may be operating in this system. Lastly, fertilization data suggest that the genes involved in hybrid male sterility may be polymorphic in one or both of the parent species. Future work will evaluate the potential role of ecological factors in the diversification of chorus frogs.

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Supporting Information

The following supporting information is available for this article:

Appendix A1. Bayesian model.

Appendix A2. Supporting Information literature cited.

Table S1. First-generation crosses (F1 hybrids and *P. feriarum*) created to assess hybrid viability, fertility, and mating success.

Table S2. Second-generation crosses created to assess hybrid fertility.

Table S3. Female *Pseudacris feriarum* tested in preference experiments.

Table S4. Results of statistical tests to determine whether tubs can be pooled within families.

Table S5. Expanded summary of previous frog hybrid fitness studies.

Supporting Information may be found in the online version of this article.

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