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Hybrid response to pathogen infection in interspecific crosses between two amphibian species (Anura: Ranidae)

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ABSTRACT

Interspecific hybridization can affect fitness-related traits, such as response to pathogens. Few experimental studies have directly addressed the effects of pathogens on host fitness in hybrid zones, particularly in animal systems. I used a system of hybridizing leopard frogs to test for differences in response to disease as a function of genetic admixture. I raised F_1 hybrid and parental genotypes between *Rana blairi* (plains leopard frog) and *Rana sphenocephala* (southern leopard frog) in replicated experimental tanks, and tested the effect of an emergent amphibian pathogen, chytrid fungi (*Batrachochytrium dendrobatidis*), on larval growth and development. Chytrid did not have a significant effect on larval survival for any genotype. Chytrids reduced body mass at metamorphosis and increased duration of the larval period for all genotypes. F_1 hybrids experienced smaller metamorphic body masses and longer larval periods than both parental genotypes, but only in chytrid environments. Significantly, more F_1 hybrids were infected than either parental species. These results indicate that interspecific hybridization may produce relatively fit hybrid genotypes in pathogen-free conditions, but hybrids suffer decreased performance in the presence of chytrids. Such results suggest that hybridization between divergent amphibian lineages may produce hybrid genotypes with a reduced ability to cope with emergent pathogens.

Keywords: amphibian, chytridiomycosis, disease, hybridization, larvae, pathogens, *Rana*.

INTRODUCTION

Hybrid zones provide ideal circumstances for the study of evolutionary and ecological phenomena (Hewitt, 1988; Harrison, 1990; Arnold, 1997), particularly the study of disease. Hybridization between species pairs offers unique opportunities to examine the complex relationship between host genotype, environmental influences and pathogen impact. Hybridization can alter values of traits related to fitness, such as response to pathogens. If hybridization results in a reduced ability to cope with infection, pathogens may eliminate susceptible recombinant genotypes (Sage *et al.*, 1986; Dupont and Crivelli, 1988; Moulia,

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1999). In this regard, hybrids may be affected by pathogens because of incompatibilities between genes responsible for disease response (Barton and Hewitt, 1985; Whitham, 1989; Moulia *et al.*, 1991; Fritz *et al.*, 1999). Hybrids also may function as disease sinks because of their potential to harbour greater pathogen abundances than parental species (Sage *et al.*, 1986; Whitham, 1989). Alternatively, genetic recombination in hybrids may not affect disease response negatively, and patterns of equivalent or higher performance for hybrids in pathogen environments may occur (Fritz *et al.*, 1994; Strauss, 1994; Arnold, 1997). Such recombinant genotypes may exhibit greater genetic variability, allowing them to cope better with infection (Fritz *et al.*, 1994, 1998). Hybridization also can have long-term effects detectable in phylogenetic analyses, potentially resulting in new taxa (Arnold, 1997; Dowling and Secor, 1997) with novel patterns of disease resistance.

The evolutionary consequences of disease on hybrid genotypes, although well studied in plants, have seldom been tested in animals (Moulia, 1999). Moreover, rarely have manipulative studies been performed that clearly test parental and hybrid genotype responses to disease (Arnold and Hodges, 1995). Progress on assessing the impact of disease on specific genetic backgrounds is possible by developing model hybrid systems. Hybridization has played a significant role in the evolution of the *Rana pipiens* North American leopard frog species complex. Consisting of more than 25 species, frogs in this group have been influential in advancing our understanding of speciation and evolutionary dynamics within hybrid zones (Hillis *et al.*, 1983; Hillis, 1988). Parapatric geographic distributions and narrow zones of hybridization are characteristic of most species pairs within the group (Sage and Selander, 1979; Hillis, 1981, 1988), although some species interact sympatrically and hybridize extensively (Parris, 2001a). Hybrid inferiority is commonly inferred in many leopard frog hybrid zones (Sage and Selander, 1979; Hillis, 1981; Frost and Platz, 1983; Kocher and Sage, 1986), but experimental data indicate that hybrids may not always be at a disadvantage and fitness may depend on the environment (Parris, 1999, 2000, 2001a,b; Parris *et al.*, 1999). Hybridization between *Rana blairi* (plains leopard frog) and *R. sphenocephala* (southern leopard frog) has been investigated thoroughly: hybrids between these two species exhibit equivalent larval fitness as parental genotypes in experimental ponds (Parris, 1999) and field enclosures (Parris, 2001c) in the absence of known disease.

Understanding host–pathogen dynamics in amphibians is urgent in the wake of alarming worldwide population declines, many of which are associated with diseases (Alford and Richards, 1999; Lips, 1999; Carey, 2000; Collins *et al.*, 2003). Infectious diseases play key roles in many amphibian population declines (Daszak *et al.*, 1999, 2000). Chytridiomycosis, caused by a chytrid fungus pathogenic to amphibians (*Batrachochytrium dendrobatidis*), has led to significant amphibian declines in North America, Central America, Spain and Australia (Berger *et al.*, 1998; Bosch *et al.*, 2001; Bradley *et al.*, 2002; Collins *et al.*, 2003). The recent discovery of the disease and its rapid spread qualify chytridiomycosis as an emerging infectious disease threatening amphibian populations worldwide (Daszak *et al.*, 1999, 2003).

I tested the hypothesis that variation in genetic structure influences response to disease in hybrid and parental leopard frogs using a manipulative common-garden experimental approach. I predicted four potential outcomes (*sensu* Fritz, 1999): (1) hybrids and parentals do not differ in larval fitness measures in chytrid environments; (2) hybrids experience more severe reductions in larval fitness relative to parental genotypes in the presence of disease; (3) if parental species differ in fitness measures, hybrid fitness is equivalent to that of one

parental species; and (4) hybrids experience heterosis in larval fitness in response to infection. Because natural hybridization is a common phenomenon among North American leopard frog species boundaries (Hillis, 1988), it is critical to understand interactions between host genotype and the environment to assess the importance of chytrid fungal infections in affecting potential declines and mediating hybrid zone evolution.

MATERIALS AND METHODS

Source populations and breeding design

I selected populations of *Rana blairi* and *R. sphenocephala* from allopatric areas in southeastern Missouri (Mississippi County; 36°43′N, 89°20′W) in shallow seasonal floodplain ponds and remnant cypress swamps. The hydroperiod of many breeding habitats in the region is regulated by groundwater connections with the Mississippi River. Agriculture (corn, rice, soybeans) dominates the surrounding habitat, with old growth bottomland hardwood and swamp forests interspersed.

I used artificial crosses on 25 April 2001 to produce offspring of *R. blairi* (BB), *R. sphenocephala* (SS) and F_1 hybrids. I collected adult frogs for crosses by hand during 22–24 April from habitats throughout the study area. Pure species status was confirmed by previously conducted nocturnal calling surveys, morphological identification and protein electrophoresis using the diagnostic isozyme loci LDH-B, PGM-1 and SDH (Hillis *et al.*, 1983; Sage, 1994; Parris *et al.*, 1999). I used a factorial crossing design to generate sibships of parental and F_1 hybrid larvae. Each of three *R. blairi* females were crossed to the same three conspecific males to produce parental genotype BB (9 half-sib families), and each of four *R. sphenocephala* females were crossed with the same four conspecific males to produce parental genotype SS (16 half-sib families). I pooled resultant sibships within parental cross types together because preliminary laboratory studies indicated no significant differences among families in response to disease (unpublished data). I produced the F_1 hybrid genotype by crossing the same *R. blairi* and *R. sphenocephala* adults used to generate the parental genotypes. Two reciprocal F_1 genotypes were generated: BS (*R. blairi* female × *R. sphenocephala* male) and SB (*R. sphenocephala* female × *R. blairi* male). Crossing three *R. blairi* females with four *R. sphenocephala* males produced the BS genotype (12 half-sib families), and crossing four *R. sphenocephala* females with three *R. blairi* males produced the SB genotype (12 half-sib families). Because of a lack of significant differences among families (unpublished data), I pooled resultant sibships within BS and SB F_1 hybrid genotypes together.

Females did not require exogenous hormone stimulation to induce ovulation. I performed crosses by stripping 20–40 eggs from one female at a time into a sperm suspension, which was prepared by macerating both testes of a male in a small volume of pond water in a petri dish. I allowed 4–7 min for fertilization, after which the sperm suspension was poured into a new petri dish and the eggs covered with fresh pond water. I then added eggs of the next female to the sperm suspension and allowed fertilization accordingly. All fertilizations were performed within 5 h. This time period has been sufficient to produce equivalent levels of fertilization success throughout the entire crossing period in previous experiments (e.g. Parris, 1999). At room temperature (24°C), most larvae hatched in 4–5 days, after which they were transferred to larger containers of pond water (1.5 litres).

Larval fitness measurements and experimental design

I raised larvae in 32 polyethylene experimental tanks (1.83 m diameter) positioned in an array at the University of Memphis Edward J. Meeman Biological Field Station (Shelby County, Tennessee; 35°22′N, 90°1′W). Tanks were exposed to natural, seasonal changes in air temperature and photoperiod. Although relatively small, experimental tanks are within the size range of small ephemeral habitats used by leopard frogs in southeastern Missouri (personal observation). I prepared the tanks in mid-April 2001 by filling them with tap water to a depth of 30 cm (750 litres), adding 1.0 kg of air-dried leaf litter collected from nearby deciduous forests, and inoculating them seven times with 500-ml aliquots of a concentrated plankton suspension collected from several nearby natural ponds. I also added three adult snails (Lymnaeidae) to graze algae on tank surfaces and facilitate nutrient turnover (Bronmark *et al.*, 1991). These initiation procedures established complex aquatic environments with self-maintaining food webs of algae and plankton for developing larvae (Parris, 1999). I added no water during the experiment, as rainfall compensated for evaporative water loss. Securely fastened and weighted lids (fibreglass screen, 1 mm mesh) were attached to each tank to provide shading and prevent colonization by feral predators and competitors. I allowed tanks to condition undisturbed for 14 days before adding larvae.

My design tested the relationship between genotype, rearing association (single-genotype groups or mixtures) and effect of the pathogen (chytrid fungus) on host performance. Treatments consisted of larvae of *R. blairi*, *R. sphenocephala* and F_1 hybrids reared separately (60 larvae per tank) or all three genotypes reared together (20 of each genotype; 60 in total per tank) in the presence or absence of chytrid. I used initial stocking densities comparable to natural *Rana* densities (60 larvae per tank = 12.5 litres per larva; Morin, 1983; Werner and Glennemeier, 1999) and those used in previous experiments (Parris, 1999, 2001b). I replicated the eight treatments four times each and randomly assigned them to the 32 tanks. Reciprocal F_1 hybrids (BS and SB) were replicated twice each within the F_1 hybrid genotype replicates. Within each cross type, all of the sibships were combined together and apportioned to the treatments. On 3 May (day 0), after reaching free-swimming (stages 25–26; Gosner, 1960), I haphazardly selected larvae and randomly added them according to the design. Larvae acclimated 7 days before the chytrid treatments started.

Exposure to chytrid fungus is known to occur in many areas of the range of leopard frogs in the USA (Morell, 1999; Morehouse *et al.*, 2003; J. Mitchell, D. Green and K. Lips, personal communications). My design, therefore, explicitly tested the response of widespread amphibian species to pathogens likely to be experienced in natural populations. I applied chytrid treatments by exposing larvae to infected adult frogs. I performed infections 2 weeks earlier in the laboratory by exposing adult *R. sphenocephala* to water baths containing infectious chytrid concentrations (7000 zoospores$\cdot$ml^{-1}; Davidson *et al.*, 2003). Chytrid was cultured in the laboratory on tryptone-gelatin hydrolysate-lactose (TGhL) agar in petri dishes according to a standardized protocol (Longcore *et al.*, 1999; Davidson *et al.*, 2003). I confirmed chytrid infections by examining formalin-fixed sloughed skin with a standard light microscope. The chytrid treatment began on 10 May by placing a chytrid-infected adult *R. sphenocephala* in a buoyant mesh cage in each treatment tank. Cages ($25 \times 20 \times 12$ cm) were constructed of untreated lumber with black flexible plastic netting securely stapled on two surfaces. Buoyancy was maintained by tubular PVC floats attached at two ends of the cage. Because chytrid can be transmitted through water (Longcore *et al.*,

1999; Pessier *et al.*, 1999; Nichols *et al.*, 2001), naive experimental larvae could be affected by contact with water from the infected animals in the cages. My design, therefore, simulated transmission by water, one of the likely modes of chytrid transmission in natural amphibian populations. A non-infected *R. sphenocephala* adult was placed in control tank cages. Cage frogs did not differ significantly in body mass among treatments ($P = 0.861$). Infected cage frogs were removed when they died. All infected cage frogs died within 9 days, at which point frogs from control tank cages were also removed.

To test if the method of pathogen introduction had a confounding effect on larval performance independent of chytrid fungi, I censused bacterial populations and primary productivity in each tank three times during the 60-day experimental period. Infected cage frog mortality could affect larval performance differently than in tanks containing control cage frogs by potentially elevating bacteria concentrations or reducing plankton growth. I tested for differences in bacteria concentrations between control and chytrid-infected tanks by assaying Gram-negative bacteria. Water samples were analysed at day 7 (initiation of pathogen treatments), day 16 (removal of control and pathogen-infected frogs) and day 49 (emergence of first metamorph). For each sample, I serially diluted 1 ml of water to 10^{-6} ml. I plated samples onto 90-mm plates containing Emb agar. Plates were incubated at 37°C for 18 h, after which I counted the number of bacterial colonies. Productivity was censused on the same days as the bacterial assays using standard methods (American Public Health Association, 1998). I used total chlorophyll and phaeophytin content as an index of primary productivity. Minimum and maximum water temperatures at the bottom of two tanks (one at each end of the array) were recorded weekly, and there were no significant differences in temperature ($P = 0.922$). Temperatures ranged from 21 ± 6°C to 33 ± 4°C over the course of the experiment.

Response variables and statistical analyses

I used proportion of individuals surviving, body mass at metamorphosis and the duration of the larval period as measures of larval performance. I considered mean values per tank as the unit of analysis because measurements from individuals within tanks were not independent. I calculated survival as the proportion of all larvae and metamorphs surviving to day 60 from those initially added to each tank. When larvae begin to metamorphose (day 49), I checked tanks daily for froglets. I defined metamorphosis by the emergence of one forelimb (Gosner, 1960) and weighed metamorphs in the laboratory after tail resorption was complete (2–4 days). Body mass at tail resorption is an accurate index of metamorphic body mass (Travis, 1984). On 29–30 June (days 60 and 61), I removed all remaining larvae from tanks with dip nets. By day 60, a minimum of 15 metamorphs was collected from every tank and, in some tanks, 100% of larvae reached metamorphosis, thus justifying the 60-day experimental period. To ensure genotype status, all individuals from the mixed-genotype treatment were euthanized, frozen and later genotyped using the isozyme locus PGM-1. I also haphazardly selected 15 metamorphs from each tank (five from each genotype in mixed-genotype treatments) to confirm infections, and to test for differences in the proportion of individuals infected among parental and hybrid genotypes. Selected animals were euthanized and fixed in 10% formalin before examination with light microscopy. Each animal was examined for 5 min, with the qualitative presence of chytrid zoospores or zoosporangia being considered an indicator of infection. I compared the proportion of individuals infected among genotypes using log-likelihood ratio goodness-of-fit tests.

I thoroughly disinfected all tanks at the end of the experiment by adding bleach (6% sodium hypochlorite) to yield a 10% solution.

I measured responses separately for each genotype in the mixed-genotype treatment. Initially, larval responses were tested for main effects of genotype, disease, mixture and their interaction with multivariate analysis of variance for the three response variables together, then with univariate analyses of variance for each response separately. Preliminary analyses indicated no significant differences between F_1 hybrid genotypes BS and SB for any larval response (see Results). I therefore combined reciprocal genotypes for the primary analyses. I interpreted multivariate effects at a significance level of 0.05 and univariate effects at 0.0125 (Bonferroni-adjusted for three response variables). Because of a significant correlation between body mass at metamorphosis and larval period length ($r = -0.52$, $P = 0.0001$), each was used as a covariate in analysis of covariance of the other. I conducted conservative pairwise tests of significance among genotype level means using Scheffé's multiple range tests. I angularly transformed survival data, and log-transformed body mass and larval period length data to increase additivity of effects and homogeneity of error variances (Sokal and Rohlf, 1995). I included all responses in analyses of (co)variance after both normality and homogeneity of error variances between genotype and disease factor levels were confirmed. I used SAS/STAT for all statistical procedures (SAS, 1990).

RESULTS

The main effects of genotype and disease, and the interaction between them, were significant in the overall multivariate analysis of variance (Table 1). Analysis of variance indicated that the proportion of larvae surviving did not make a contribution to the significant multivariate effects (Tables 1 and 2; Fig. 1A). Reciprocal F_1 hybrid genotypes did not differ significantly in terms of survival ($F_{1,8} = 0.05$, $P = 0.8221$). There were, however, strong effects of chytrid on the remaining responses (Table 1). Body mass at metamorphosis decreased (Fig. 1B), and larval period length increased (Fig. 1C), significantly in the presence of chytrid. Genotypes did not differ significantly in body mass, but F_1 hybrids had longer larval period lengths than either parental genotype (Table 1; Figs. 1B and 1C). Reciprocal F_1 hybrid genotypes did not differ significantly in body mass ($F_{1,7} = 0.40$, $P = 0.5479$) or larval period length ($F_{1,7} = 0.50$, $P = 0.5008$). The main effects of genotype and disease were secondary to their interaction (Table 1). In the absence of chytrid, parental and F_1 hybrid genotypes did not differ significantly in metamorphic body mass or larval period length. However, in the presence of chytrid, F_1 hybrids experienced a 28% decrease in body mass and a 5% increase in larval period length relative to when reared in disease-free conditions (Table 2; Figs. 1B and 1C). Larval responses were not significantly affected by the mixture main effect (Table 1).

Examination of metamorph samples indicated that significantly more F_1 hybrids were infected relative to parental genotypes BB ($G_1 = 14.84$, $P = 0.0001$) and SS ($G_1 = 12.66$, $P = 0.0004$). Parental genotypes did not differ significantly in infection rates ($G_1 = 0.09$, $P = 0.7602$). Infection rates of metamorphs samples were $93.3 \pm 6.2\%$ (mean $\pm$ S.D.) for F_1 hybrids, $75.8 \pm 5.0\%$ for BB and $77.5 \pm 5.0\%$ for SS. No animals from disease-free tanks were infected. Water sample analyses revealed no significant differences between control and chytrid-infected treatments across the three sample dates for bacterial densities (control $= 47.40 \pm 14.2$ colonies $\cdot$ ml^{-1}, infected $= 49.57 \pm 13.3$ colonies $\cdot$ ml^{-1}; $F_{1,46} = 0.54$, $P = 0.4673$; repeated-measures analysis of variance) or productivity (control $= 39.45 \pm 9.31$

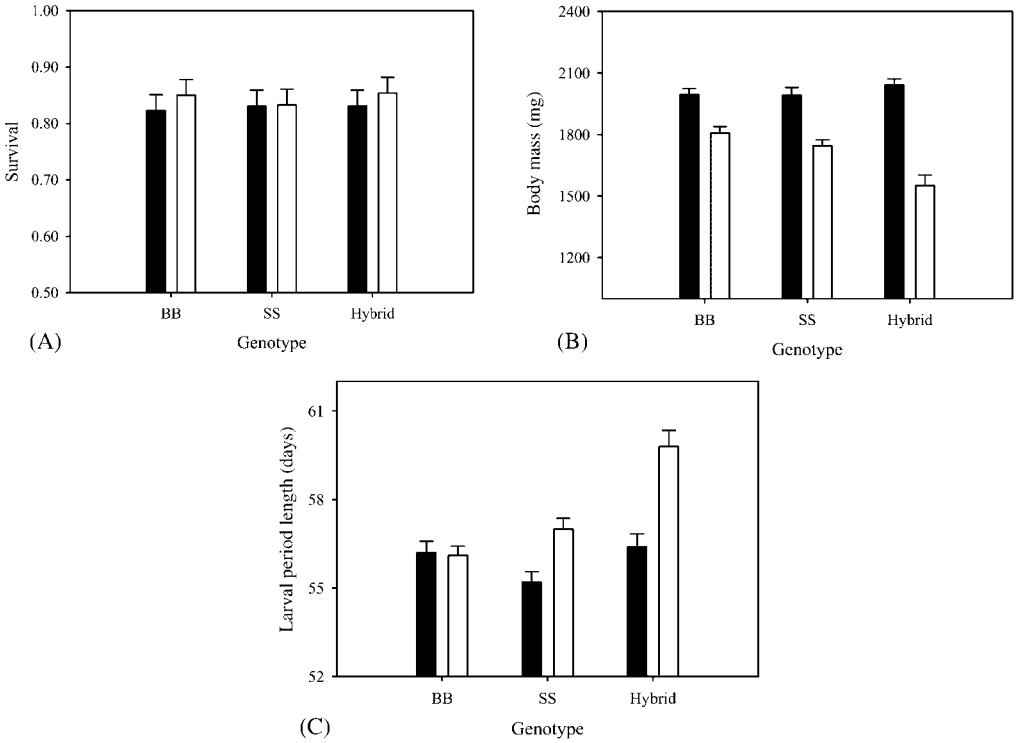

Fig. 1. (A) Proportion of individuals surviving, (B) body mass at metamorphosis and (C) larval period length for *Rana blairi* (BB), *R. sphenocephala* (SS) and F_1 hybrids reared in experimental tanks in the absence (control, ■) or presence (disease, □) of chytrid fungus. Values plotted are least-squares treatment means (corrected for larval period length in (B) and for body mass in (C)) ± 1 S.E.

$\mu g \cdot l^{-1}$, infected = 41.61 ± 9.30 $\mu g \cdot l^{-1}$; $F_{1,46} = 1.02$, $P = 0.3168$; repeated-measures analysis of variance).

DISCUSSION

The results of this study demonstrate that pathogens can have strong effects on larval life-history traits, and could affect the outcome of hybridization between *Rana blairi* and *R. sphenocephala*. Although chytrid fungus reduced larval performance for all genotypes, the significant genotype × disease interaction indicates that F_1 hybrids suffered greater reductions in larval fitness measures in the presence of disease. Higher rates of detected infection for hybrids relative to both parental genotypes further suggest that F_1 hybrids are at a selective disadvantage in chytrid environments. In the absence of chytrid, hybrids performed as well as their parental genotypes in terms of survival and body mass at metamorphosis, but experienced significantly longer larval period lengths. However, most of the difference in larval period length among genotypes occurred in disease environments, suggesting that in disease-free conditions F_1 hybrids may be relatively successful. Such high relative fitness of hybrids in Missouri was corroborated by Parris (1999, 2000, 2001a,b) in a series of common-garden experiments. The severe decreases in hybrid growth and

Table 1. Summary of multivariate analysis of variance (MANOVA), univariate analysis of variance (ANOVA) of proportion of individuals surviving and analyses of covariance (ANCOVA) for body mass at metamorphosis and duration of the larval period of *Rana blairi*, *R. sphenocephala* and F_1 hybrid larvae reared in the presence or absence of chytrid fungus in single-genotype populations and mixtures in experimental tanks

MANOVA: survival, body mass, larval period length				
Source	Wilks' λ	d.f.	*F*	*P*
Genotype	0.338	6,68	8.17	<0.0001
Disease	0.152	3,34	63.28	<0.0001
Mixture	0.926	3,34	0.90	0.4510
Genotype × disease	0.360	6,68	7.55	<0.0001
Genotype × mixture	0.960	6,68	0.23	0.9643
Disease × mixture	0.974	3,34	0.30	0.8232
Genotype × mixture × disease	0.833	6,68	1.08	0.3815

ANOVA: survival					
Response	Source	d.f.	MS	*F*	*P*
Survival	Genotype	2	0.001	0.10	0.9057
	Disease	1	0.012	0.70	0.4093
	Mixture	1	0.008	0.50	0.4841
	Genotype × disease	2	0.002	0.10	0.9060
	Genotype × mixture	2	0.002	0.10	0.9040
	Disease × mixture	1	0.006	0.35	0.5574
	Genotype × mixture × disease	2	0.002	0.12	0.8851
	Error	36	0.017		

ANCOVA: body mass and larval period

Response	Source	d.f.	MS	F	P
Body mass	Genotype	2	0.001	4.02	0.0467
	Disease	1	0.037	100.42	<0.0001
	Mixture	1	0.001	1.74	0.1954
	Genotype × disease	2	0.004	10.87	0.0002
	Genotype × mixture	2	<0.001	0.28	0.7568
	Disease × mixture	1	<0.001	0.30	0.5872
	Genotype × mixture × disease	2	<0.001	0.40	0.6735
	Larval period (covariate)	1	0.023	61.25	<0.0001
	Error	35	<0.001		
Larval period	Genotype	2	0.001	22.78	<0.0001
	Disease	1	0.001	9.51	0.0040
	Mixture	1	<0.001	0.72	0.4020
	Genotype × disease	2	<0.001	11.14	0.0002
	Genotype × mixture	2	<0.001	0.16	0.8525
	Disease × mixture	1	<0.001	0.07	0.7910
	Genotype × mixture × disease	2	<0.001	2.11	0.1359
	Body mass (covariate)	1	0.002	38.62	<0.0001
	Error	35	<0.001		

Note: Body mass and larval period length were used as covariates on each other in the analyses of covariance. See text for explanation.

Table 2. Summary of uncorrected means (± 1 S.E.) of proportion of individuals surviving, body mass at metamorphosis (mg) and larval period length (days) for *Rana blairi*, *R. sphenocephala* and F_1 hybrids reared in the absence (control) or presence (disease) of chytrid fungus, either alone or all three genotypes combined (mixed)

Genotype	Treatment	Survival	Body mass	Larval period
F_1	Control – alone	0.86 ± 0.039	2048.3 ± 42.1	57.1 ± 0.43
F_1	Control – mixed	0.80 ± 0.039	2029.5 ± 42.1	56.2 ± 0.43
F_1	Disease – alone	0.86 ± 0.039	1578.0 ± 42.1	59.3 ± 0.43
F_1	Disease – mixed	0.85 ± 0.039	1607.3 ± 42.1	59.4 ± 0.43
BB	Control – alone	0.83 ± 0.039	1983.3 ± 42.1	56.6 ± 0.43
BB	Control – mixed	0.81 ± 0.039	1995.8 ± 42.1	56.2 ± 0.43
BB	Disease – alone	0.86 ± 0.039	1745.0 ± 42.1	55.8 ± 0.43
BB	Disease – mixed	0.84 ± 0.039	1843.0 ± 42.1	56.2 ± 0.43
SS	Control – alone	0.84 ± 0.039	1944.0 ± 42.1	55.1 ± 0.43
SS	Control – mixed	0.83 ± 0.039	1995.3 ± 42.1	55.7 ± 0.43
SS	Disease – alone	0.84 ± 0.039	1748.5 ± 42.1	57.2 ± 0.43
SS	Disease – mixed	0.83 ± 0.039	1743.5 ± 42.1	56.4 ± 0.43

Note: All treatment combinations were replicated four times, and the experiment was concluded at day 60.

development in the current study demonstrate that patterns of relative fitness can be altered by chytrid fungus, and thus pathogens may play an important role in determining the evolutionary trajectory of hybrid genotypes between *R. blairi* and *R. sphenocephala*.

Batrachochytrium dendrobatidis may impact populations of leopard frogs by affecting growth and development rather than survival. Although the direct effects of differential survival on fitness are obvious (Endler, 1986), I did not observe a significant effect of *B. dendrobatidis* on survival, suggesting that chytridiomycosis may not have a direct effect on larval survival in this system of leopard frogs. However, in experimental tank environments with constant water levels and without interspecific competitors or predators, larvae were sheltered from sources of mortality present in natural populations. My design, therefore, precluded detection of survivorship impacts that larvae could experience as a result of sub-lethal chytrid infections. Moreover, chytrid infections can produce disfigurement of the keratinized mouthparts of amphibian larvae, but Fellers *et al.* (2001) documented that chytrid-induced mouthpart abnormalities may not have a negative impact on larval fitness. Although not investigated in the present study, an inability to obtain sufficient resources would probably have serious repercussions for juvenile recruitment into the terrestrial environment because larvae must obtain enough resources to reach a certain minimal body size required for metamorphosis (Wilbur and Collins, 1973). Reduced F_1 hybrid metamorphic body mass and increased larval period length in chytrid environments can have important consequences on lifetime fitness. Small body mass at metamorphosis may lead to decreased juvenile survival, later age and smaller size at first reproduction, and lower reproductive success (Berven and Gill, 1983; Smith, 1987; Semlitsch *et al.*, 1988). Furthermore, rapid developmental rates are advantageous for leopard frogs because they allow larvae to metamorphose quickly and escape desiccation in ephemeral environments and minimize prolonged exposure to aquatic predators and disease (Newman, 1988; Denver *et al.*, 1998). Thus, the chytrid impacts on larval life-history traits I measured are likely to reflect differences important to lifetime fitness (De Jong, 1994).

It is important to note that rearing mixture did not have a significant effect on any larval response. Thus, all three genotypes were competitively equivalent, which conflicts with the results of Parris (2001a), who found that F_1 hybrid and parental genotypes metamorphosed at a larger size when reared alone relative to when combined in field enclosures. Such a discrepancy may be due to different original source populations of animals for the two studies and cautions against inferring hybrid zone dynamics using information from a single location. Furthermore, the lack of a significant disease × mixture interaction suggests that the impact of chytrids on larval leopard frogs can be predicted based on effects in single-genotype groups. The genotypic composition of larval populations of *R. blairi* and *R. sphenocephala* in sympatric areas is spatially and temporally heterogeneous, often consisting of complex mixtures of hybrid and parental genotypes (Sage, 1994). Statistically equivalent chytrid effects across all mixture treatments in my experiment suggest that environmental effects on hybrid resistance, such as biotic components of larval guilds, may not be important in this system.

The number of replicated families generated within each genotype was high for studies of vertebrate hybrid zones (24 F_1 hybrids, 9 BB, 16 SS), yet is likely not to encompass the full range of genetic variation in disease response in this leopard frog hybrid system. The limited number of adults used to create the larvae also limits the generality of my results. Nevertheless, the lack of a significant difference between reciprocal F_1 hybrid genotypes in the current study, coupled with previous findings of minimal variation among half-sib families in response to disease (unpublished data), suggests that the observed responses may represent overall genotypic responses.

Hybrid response to disease may vary according to degree of genetic admixture because different genetic processes that influence the response may be important in each class. In the present study, only primary generation (F_1) hybrids were used. Thus, disease response traits were inherited from both parental species without the opportunity for genetic recombination. If recombination disrupts co-adapted gene complexes, breakdown in resistance may not be manifested until the backcross or F_2 hybrid generations (Fritz *et al.*, 1996; Moulia, 1999). By demonstrating reduced performance of primary generation hybrids in chytrid environments, the results of the current study clearly indicate that dysgenesis occurs in F_1 hybrids. Because breakdown did not occur in disease-free environments, the incompatibilities between parental genomes could have involved genes responsible for disease response. Alternatively, because many frogs lack a cell-mediated immune response to chytrid fungus, there is a greater likelihood that hybrids may suffer dysgenesis in their antimicrobial peptide skin response to infection (Rollins-Smith *et al.*, 2002).

Although the fitness component estimates used in this study are reflective of lifetime fitness for amphibians, a synthetic view of chytrid impacts should take into account selection acting on a suite of other larval traits and during the terrestrial juvenile and adult life stages. In disease-free experimental environments, Parris (2000) demonstrated that hybrids between *R. blairi* and *R. sphenocephala* were at a severe disadvantage in a desiccating larval environment. Because ephemeral habitats are the predominant breeding environments for most leopard frogs, chytrid infections could exacerbate selection against hybrids and potentially reduce the frequency of interspecific hybridization. Disease-enhanced selection against hybridization also could occur after metamorphosis: reduced hybrid male fertility (Parris *et al.*, 1999) and metamorphic jumping ability (Semlitsch *et al.*, 1999) has been observed for certain hybrid leopard frog genotypes in disease-free conditions. Thus, if disease resistance mechanisms are similar for all stages of the complex life

cycle, leopard frog hybrids may be at a significant disadvantage in chytrid environments. It is important to note that I only examined hybridization–disease dynamics between two species of leopard frogs, and only used animals from one location in the hybrid zone. Thus, the generality of my results to all instances of hybridization between leopard frog species pairs needs to be tested.

Relatively few studies have examined the constraining impact of pathogens on animal hybrid zone evolution. The present results suggest that pathogens, by reducing the relative fitness of interspecific hybrids, potentially could facilitate evolutionary divergence between closely related species. It is crucial that more studies incorporate replicated multifactorial experiments to discern the complex interactions between host genotype and disease and arrive at a more general predictive theory of hybrid zone evolution.

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REFERENCES

Alford, R.A. and Richards, S.J. 1999. Global amphibian declines: a problem in applied ecology. *Annu. Rev. Ecol. Syst.*, **30**: 133–165.

American Public Health Association, American Water Works Association and the Water Pollution Control Federation. 1998. *Standard Methods for the Examination of Water and Wastewater*, 20th edn. New York: American Public Health Association.

Arnold, M.L. 1997. *Natural Hybridization and Evolution*. Oxford: Oxford University Press.

Arnold, M.L. and Hodges, S.A. 1995. Are natural hybrids fit or unfit relative to their parents? *Trends Ecol. Evol.*, **10**: 67–71.

Barton, N.H. and Hewitt, G.M. 1985. Analysis of hybrid zones. *Annu. Rev. Ecol. Syst.*, **16**: 113–148.

Berger, L., Speare, R., Daszak, P. *et al.* 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. USA*, **95**: 9031–9036.

Berven, K.A. and Gill, D.E. 1983. Interpreting geographic variation in life history traits. *Am. Zool.*, **23**: 85–97.

Bosch, J., Martínez-Solano, I. and García-París, M. 2001. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biol. Conserv.*, **97**: 331–337.

Bradley, G.A., Rosen, P.C., Sredl, M.J., Jones T.R. and Longcore, J.E. 2002. Chytridiomycosis in native Arizona frogs. *J. Wildlife Dis.*, **38**: 206–212.

Bronmark, C., Rundle, S.D. and Erlandsson, J. 1991. Interactions between freshwater snails and tadpoles: competition and facilitation. *Oecologia*, **87**: 8–18.

Carey, C. 2000. Infectious disease and worldwide declines of amphibian populations, with comments on emerging diseases in coral reef organisms and in humans. *Environ. Health Persp.*, **108**: 143–150.

Collins, J.P., Brunner, J.L., Miera, V. *et al.* 2003. Ecology and evolution of infectious disease. In *Amphibian Conservation* (R.D. Semlitsch, ed.), pp. 137–151. Washington, DC: Smithsonian Institution Press.

Daszak, P., Berger, L., Cunningham, A.A. *et al.* 1999. Emerging infectious diseases and amphibian population declines. *Emerg. Infect. Dis.*, **5**: 735–748.

Daszak, P., Cunningham, A.A. and Hyatt, A.D. 2000. Emerging infectious diseases of wildlife: threats to biodiversity and human health. *Science*, **287**: 443–449.

Daszak, P., Cunningham, A.A. and Hyatt, A.D. 2003. Infectious disease and amphibian population declines. *Diversity and Distributions*, **9**: 141–150.

Davidson, E.W., Parris, M.J., Collins, J.P. *et al.* 2003. Pathogenicity and transmission of chytridiomycosis in tiger salamanders (*Ambystoma tigrinum*). *Copeia*, **2003**: 601–607.

De Jong, G. 1994. The fitness of fitness concepts and the description of natural selection. *Q. Rev. Biol.*, **69**: 3–29.

Denver, R., Mirhadi, N. and Phillips, M. 1998. Adaptive plasticity in amphibian metamorphosis: response of *Scaphiopus hammondii* tadpoles to habitat desiccation. *Ecology*, **79**: 1859–1872.

Dowling, T.E. and Secor, C.L. 1997. The role of hybridization in the evolutionary diversification of animals. *Annu. Rev. Ecol. Syst.*, **28**: 593–619.

Dupont, F. and Crivelli, A.J. 1988. Do parasites confer a disadvantage to hybrids? *Oecologia*, **75**: 587–592.

Endler, J.A. 1986. *Natural Selection in the Wild*. Princeton, NJ: Princeton University Press.

Fellers, G.M., Green, D.E. and Longcore, J.E. 2001. Oral chytridiomycosis in the mountain yellow-legged frog (*Rana muscosa*). *Copeia*, **2001**: 945–953.

Fritz, R.S. 1999. Resistance of hybrid plants to herbivores: genes, environment, or both? *Ecology*, **80**: 382–391.

Fritz, R.S., Nichols-Orians, C.M. and Brunsfeld, S.J. 1994. Interspecific hybridization of plants and resistance to herbivores: hypotheses, genetics, and variable responses in a diverse community. *Oecologia*, **97**: 106–117.

Fritz, R.S., Roche, B.M., Brunsfeld, S.J. and Orians, C.M. 1996. Interspecific and temporal variation in herbivore responses to hybrid willows. *Oecologia*, **108**: 121–129.

Fritz, R.S., Roche, B.M. and Brunsfeld, S.J. 1998. Genetic variation in resistance of hybrid willows to herbivores. *Oikos*, **83**: 117–128.

Fritz, R.S., Moulia, C. and Newcombe, G. 1999. Resistance of hybrid plants and animals to herbivores, pathogens, and parasites. *Annu. Rev. Ecol. Syst.*, **30**: 565–591.

Frost, J.S. and Platz, J.E. 1983. Comparative assessment of modes of reproductive isolation among four species of leopard frogs (*Rana pipiens* complex). *Evolution*, **37**: 66–78.

Gosner, K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, **16**: 183–190.

Harrison, R.G. 1990. Hybrid zones: windows on evolutionary processes. In *Oxford Surveys in Evolutionary Biology*, Vol. 7 (J. Antonovics and D.J. Futuyma, eds), pp. 69–128. Oxford: Oxford University Press.

Hewitt, G.M. 1988. Hybrid zones: natural laboratories for evolutionary studies. *Trends Ecol. Evol.*, **3**: 158–167.

Hillis, D.M. 1981. Premating isolating mechanisms among three species of the *Rana pipiens* complex in Texas and southern Oklahoma. *Copeia*, **1981**: 312–319.

Hillis, D.M. 1988. Systematics of the *Rana pipiens* complex: puzzle and paradigm. *Annu. Rev. Ecol. Syst.*, **19**: 39–63.

Hillis, D.M., Frost, J.S. and Wright, D.A. 1983. Phylogeny and biogeography of the *Rana pipiens* complex: a biochemical evaluation. *Syst. Zool.*, **32**: 132–143.

Kocher, T.D. and Sage, R.D. 1986. Further genetic analyses of a hybrid zone between leopard frogs (*Rana pipiens* complex) in central Texas. *Evolution*, **40**: 21–33.

Lips, K.R. 1999. Mass mortality and population declines of anurans at an upland site in western Panama. *Conserv. Biol.*, **13**: 117–125.

Longcore, J.E., Pessier, A.P. and Nichols, D.K. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia*, **91**: 219–227.

Morehouse, E.A., James, T.Y., Ganley A.R.D. *et al.* 2003. Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. *Mol. Ecol.*, **12**: 395–403.

Morell, V. 1999. Are pathogens felling frogs? *Science*, **284**: 728–731.

Morin, P.J. 1983. Predation, competition, and the composition of larval anuran guilds. *Ecol. Monogr.*, **53**: 119–138.

Moulia, C. 1999. Parasitism in plant and animal hybrids: are facts and fates the same? *Ecology*, **80**: 392–406.

Moulia, C., Aussel, J.P., Bonhomme, F., Neilsen, J.T. and Renaud, F. 1991. Wormy mice in a hybrid zone: a genetic control of susceptibility to parasite infection. *J. Evol. Biol.*, **4**: 679–687.

Newman, R.A. 1988. Adaptive plasticity in development of *Scaphiopus couchii* tadpoles in desert ponds. *Evolution*, **42**: 774–783.

Nichols, D.K., Lamirande, E.W., Pessier, A.P. and Longcore, J.E. 2001. Experimental transmission of cutaneous chytridiomycosis in dendrobatid frogs. *J. Wildlife Dis.*, **37**: 1–11.

Parris, M.J. 1999. Hybridization in leopard frogs (*Rana pipiens* complex): larval fitness components in single-genotype populations and mixtures. *Evolution*, **53**: 1872–1883.

Parris, M.J. 2000. Experimental analysis of hybridization in leopard frogs (Anura: Ranidae): larval performance in desiccating environments. *Copeia*, **2000**: 11–19.

Parris, M.J. 2001a. High larval performance of leopard frog hybrids: effects of environment-dependent selection. *Ecology*, **82**: 3001–3009.

Parris, M.J. 2001b. Hybridization in leopard frogs (*Rana pipiens* complex): variation in interspecific hybrid larval fitness components along a natural contact zone. *Evol. Ecol. Res.*, **3**: 91–105.

Parris, M.J. 2001c. Hybridization in leopard frogs (*Rana pipiens* complex): terrestrial performance of newly metamorphosed hybrid and parental genotypes in field enclosures. *Can. J. Zool.*, **79**: 1552–1558.

Parris, M.J., Semlitsch, R.D. and Sage, R.D. 1999. Experimental analysis of the evolutionary potential of hybridization in leopard frogs (Anura: Ranidae). *J. Evol. Biol.*, **12**: 662–671.

Pessier, A.P., Nichols, D.K., Longcore, J.E. and Fuller, M.S. 1999. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). *J. Vet. Diag. Invest.*, **11**: 194–199.

Rollins-Smith, L.A., Doersam, J.K., Longcore, J.E. *et al.* 2002. Antimicrobial peptide defenses against pathogens associated with global amphibian declines. *Develop. Comp. Immunol.*, **26**: 63–72.

Sage, R.D. 1994. The distribution and genetic study of the leopard frogs (*Rana pipiens* complex). In *Report to the Missouri Department of Conservation*, pp. 1–42. Jefferson City, MO: Missouri Department of Conservation.

Sage, R.D. and Selander, R.K. 1979. Hybridization between species of the *Rana pipiens* complex in central Texas. *Evolution*, **33**: 1069–1088.

Sage, R.D., Heyman, D., Lim, K.C. and Wilson, A.C. 1986. Wormy mice in a hybrid zone. *Nature*, **324**: 60–63.

SAS Institute, Inc. 1990. *SAS / STAT User's Guide*, Version 6, 4th edn. Cary, NC: SAS.

Semlitsch, R.D., Scott, D.E. and Pechmann, J.H.K. 1988. Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. *Ecology*, **69**: 184–192.

Semlitsch, R.D., Pickle, J., Parris, M.J. and Sage, R.D. 1999. Jumping performance and short-term repeatability of newly metamorphosed hybrid and parental leopard frogs (*Rana sphenocephala* and *Rana blairi*). *Can. J. Zool.*, **77**: 748–754.

Smith, D.C. 1987. Adult recruitment in chorus frogs: effects of size and date at metamorphosis. *Ecology*, **68**: 344–350.

Sokal, R.R. and Rohlf, F.J. 1995. *Biometry*. New York: W.H. Freeman.
Strauss, S.Y. 1994. Levels of herbivory and parasitism in host hybrid zones. *Trends Ecol. Evol.*, **9**: 209–214.
Travis, J. 1984. Anuran size at metamorphosis: experimental test of a model based on intraspecific competition. *Ecology*, **65**: 1155–1160.
Werner, E.E. and Glennemeier, K.S. 1999. Influence of forest canopy cover on the breeding pond distributions of several amphibian species. *Copeia*, **1999**: 1–12.
Whitham, T.G. 1989. Plant hybrid zones as sinks for pests. *Science*, **244**: 1490–1493.
Wilbur, H.M. and Collins, J.P. 1973. Ecological aspects of amphibian metamorphosis. *Science*, **182**: 1305–1314.