

PRE AND POST ZYGOTIC FITNESS COMPONENTS OF HYBRIDIZATION IN  
SPADEFoot TOADS

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## **ABSTRACT**

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Pre and post zygotic fitness components of hybridization in spadefoot toads.  
(Under the direction of Karin S. Pfennig)

When hybrids are unfit, selection favors the evolution of pre-mating barriers that impede gene flow and promote population differentiation. Hybrid fitness, however, is complex and may vary across life-stages. Additionally, the costs may differ between species. Using spadefoot toads I examined pre- and post-zygotic fitness components of hybridization. First, I focused on two post-mating pre-zygotic barriers: fertilization success and clutch size. Fertilization success did not differ between conspecific and heterospecific males. Average clutch size did not differ as a result of heterospecific pairing. Therefore, species-specific gamete recognition is absent between these species. Second, I investigated post-zygotic fitness components of hybrid growth and survival in the period immediately following metamorphosis. Data suggests that while hybridization may be beneficial in early development, condition immediately following metamorphosis may lead to decreased survival over time. Hybrids were intermediate in size, suggesting one species may benefit more than the other.

For my dad

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## **CHAPTER 1**

### **Introduction**

Biological species remain distinct due to isolating mechanisms blocking gene flow between interspecific groups (Coyne and Orr, 2004). These barriers to gene flow can occur either before or after mating and are essential for maintaining separate species. Often, incipient species do not exchange genes due to pre-mating barriers that prevent mating from occurring (reviewed in Servedio and Noor, 2003; Coyne and Orr, 2004). These can include temporal, spatial and behavioral barriers. Alternatively, when pre-mating barriers are not present, hybridization can still be disfavored due to post mating barriers. Interspecific matings can fail post mating in two general ways: pre-zygotically due to problems preceding fertilization, or post-zygotically due to failures following fertilization. Post-zygotic barriers can be either intrinsic or extrinsic. Intrinsic barriers reflect developmental problems that have arisen due to genetic incompatibilities and are independent of environment (Coyne, 1992). Extrinsic barriers are environmentally dependent and occur when hybrids inherit phenotypes that results in lower fitness (Schulter, 2000).

Species that come into secondary contact after a period of isolated divergence may experience hybridization. When hybridization between species does occur, hybrid fitness plays a critical role in the evolutionary outcome of speciation. When hybrid fitness is low, mechanisms evolve that prevent mating from occurring (i.e. pre-mating barriers). This process is termed reinforcement (Dobzhansky, 1940). Heterospecific matings often produce

hybrids with low fitness (Hatfield and Schuler, 1999; Naisbit et al., 2001), but this is not always the case. Hybridization can be beneficial in some systems (Arnold, 1997; Arnold and Hodges, 1995; Semlitsch and Reyer, 1992). It is likely that hybridization is a complex process resulting in different levels of fitness depending on the environment, which species is maternal, and what fitness measures are being recorded (Arnold and Hodges, 1995). For example, some hybrids experience an increased performance in the larval stage, but decreased fecundity in adults (Parris et al., 199; Simovich, 1985; Simovich et al., 1991). The identity of the maternal species can also affect hybrid fitness. When hybrid fitness is intermediate relative to the parental species, selection may favor hybridization for one species, but not the other (Pfennig and Simovich, 2002). To gain a complete understanding of the affects of hybridization, several measures of fitness must be examined during the organism's lifespan; with this type of analysis, we can examine costs and benefits of hybridization to each species, and identify critical components that may cause strong selection for or against hybridization. Below I outline the study system used in my thesis research.

### *Study System*

*Spea bombifrons*, the plains spadefoot toad, and *Spea multiplicata*, the Mexican spadefoot toad, co-occur in several regions of the southwestern United States, including portions of Arizona, New Mexico and Texas (Stebbins, 2003). They develop extremely rapidly, allowing them to inhabit a range of ponds, from extremely ephemeral to semi-permanent.

*Spea bombifrons* and *S. multiplicata* make an ideal system for studying the complexities of hybridization for several reasons. First, hybridization occurs naturally in the wild (Forester, 1975; Sattler, 1985; Simovich, 1985; Pfennig and Simovich, 2002). Hybridization levels have been measured to be as high as 40% in some ponds, with many backcross individuals identified, suggesting introgression has occurred between these two species (Pfennig and Simovich, 2002; Simovich, 1985; Sattler, 1985). In the past few decades, hybridization rates have declined, possibly due to reinforcement (Pfennig, 2003). This is especially interesting in light of their evolutionary relationship to one another. *S. bombifrons* and *S. multiplicata* are the most divergent species within the genus *Spea* (Wiens and Titus, 1991). The decline in hybridization rates implies that some pre-mating barriers have evolved to stem introgression of these two species in areas of sympatry (Pfennig, 2003).

Second, toads are amenable to both field and laboratory studies. While these toads are rather elusive in the field, spending 9-10 months estivating underground, during the breeding season, they can easily be found around ponds following heavy rains (Bragg, 1965). In the laboratory, these two species will readily form breeding pairs with heterospecifics and conspecifics alike when given hormones to induce mating. All the animals used in this study were field caught and have been maintained in a lab at the University of North Carolina, Chapel Hill, for 1-5 years.

Finally, because of the relative ease of field and lab studies, much is already known about the fitness of hybrids as tadpoles and as sexually mature adults. Hybrid tadpoles have an intermediate development time relative to pure species types (Simovich, 1985; Simovich et al., 1991). Hybrid tadpoles with maternal *S. bombifrons* develop faster than pure species *S. bombifrons* tadpoles, conferring a survival advantage in highly ephemeral ponds (Simovitch,

1985; Simovitch et al., 1991; Pfennig and Simovich, 2002). A recent study by Pfennig (2008), showed that female *S. bombifrons* are more likely to hybridize with male *S. multiplicata* in low-water conditions than in high water conditions. In ephemeral pond conditions, the alteration of development times that results from hybridization may cause less selection against hybridization in *S. bombifrons*. This implies a benefit for *S. bombifrons* under certain environment conditions (Pfennig and Simovich, 2002). Regardless of the maternal parent, hybrids suffer fitness costs in terms of reduced fecundity or sterility when compared to pure-species types (Simovitch, 1985; Simovitch et al., 1991). Females are less fecund, with half as many eggs as pure types, and males are thought to be completely sterile in Arizona populations (Simovich, 1985) and partially to completely sterile in some Texas populations (Forester, 1975). Even though hybrids suffer severely reduced fecundity as adults, hybridization may still be beneficial in ephemeral ponds. There may be a tradeoff between survival with impaired reproductive fitness versus 100% mortality, and thus no offspring reaching reproductive maturity (Pfennig and Simovich, 2002; Pfennig, 2003).

Even though there has been extensive research on the tadpole and adult stages of the spadefoot toad life cycle, little attention has been paid to the period spanning metamorphosis to sexual maturity. In particular, little attention has been given to the growth period immediately following metamorphosis that lasts 4-6 weeks until the juveniles burrow underground for the winter.

## **Objectives**

In my thesis research, I examined the role of post-mating pre-zygotic barriers in heterospecific matings of *Spea bombifrons* and *Spea multiplicata* to determine whether

hybrid offspring suffered post-zygotic fitness deficits in early post-metamorphic growth and survival. Ultimately, this study aims to emphasize the complexity that exists in a hybrid system, illustrating when and where barriers form to reproduction, with emphasis on the critical phase of juvenile growth.

In Chapter 2, I specifically examined whether females alter clutch size when paired with a heterospecific versus conspecific male. I also examined the relative fertilization success of heterospecific versus conspecific males. I suggest that no post-mating pre-zygotic barriers are active between these two species.

In Chapter 3, I created growth and survival curves for the first six weeks post-metamorphosis to examine fitness consequences of hybridization between these two spadefoot species. In my survival analysis, I also examine the role of size and age at metamorphosis in predicting survival. I suggest that hybrid fitness depends on which species is the maternal parent, and overall, that strong reductions of fitness are present in early post-metamorphic growth.

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## CHAPTER 2

### Fertilization success in hybrids and pure species types

#### Abstract

Hybrid fitness is crucial to understanding how new species are formed and how species barriers are maintained. Hybrid fitness is complex and can vary depending on the fitness components measured. Isolated populations diverge due to drift, mutation or selection. Upon secondary contact, this divergence could create barriers to fertilization. Recent evidence shows that proteins and genes associated with gamete fertilization are among some of the most rapidly evolving. In populations that reestablish contact, this divergence could cause lower fertilization success between heterospecific pairs. This study focuses on the role of post-mating pre-zygotic barriers in two spadefoot toad species, (*Spea bombifrons* and *S. multiplicata*), that have recently come into secondary contact. Our results suggest that there are no barriers to fertilization success between these two species. Clutch size was not altered when mating with a heterospecific male, and fertilization success did not differ between conspecific and heterospecific males. Our data suggest that female spadefoot toads do not suffer an immediate fitness loss due to lower fertilization success when paired with a heterospecific.

## **Introduction**

Reproductive isolation is the definitive characteristic that determines species identity, according to the biological species concept (Mayr, 1942). Therefore, understanding why and when mis-matings occur between species can provide us with vital information on the process of speciation.

Isolated populations can diverge due to mutation, drift and selection (Coyne and Orr, 2004). When isolated populations come back into secondary contact, hybridization may occur and the fitness of the hybrids depends on the divergence that occurred in these populations while they were isolated. This divergence could cause gametic or other genetic incompatibility, as well as behavioral incongruence (Coyne and Orr, 2004). The outcome of secondary contact critically depends on the fitness of hybrid offspring. If hybrids are unfit, selection may favor pre-mating isolating barriers that reduce gene flow between the populations (Howard, 1993; Gerhardt, 1994; Noor, 1995; Pfennig, 2000; Servedio and Noor, 2003; Coyne and Orr, 2004).

Alternatively, if pre-mating barriers are absent, hybrids may be unfit due to post-mating barriers. Post-mating barriers can occur prior to fertilization (i.e. pre-zygotic), or following fertilization (i.e. post-zygotic). Pre-zygotic barriers can include the following: gametic incompatibility (Lessios and Cunningham, 1990; Palumbi and Metz, 1991), mechanical failure or fatal interactions of sperm with the female reproductive tract (Coyne and Orr, 2004). Additionally, if one species produces fewer sperm than another, sperm limitation could be a barrier (Levitan, 2002). Post-zygotic barriers may cause hybrid sterility or lethality, and can occur either intrinsically (genetic incompatibility) or extrinsically (failures in the environment).

Hybrids are not always unfit, however (Arnold, 1997). For example, in species that are sperm or pollen limited hybridization may be beneficial if heterospecific males produce more or better quality sperm that is successfully recognized by female eggs (Humphrey, 2006). If pre-zygotic barriers are present, however, the eggs may not be able to recognize the sperm, and hybridization is selected against. There is significant experimental evidence suggesting that extremely rapid evolution of traits and proteins related to fertilization occurs between species (Gavrilets, 2002). Testing populations that are known to hybridize for the presence or absence of pre-zygotic barriers has the potential to provide critical insight on the speciation process occurring between them. If pre-zygotic barriers are present, hybridization should be disfavored, and the speciation will become complete. However, if pre-zygotic barriers are absent, introgression between the two populations may continue, unless hybrids are selected against post-zygotically.

This study uses two measures of fitness, clutch size and fertilization success, to assess whether pre-zygotic barriers play a role in two naturally hybridizing species of spadefoot toads, *S. bombifrons* and *S. multiplicata*. Specifically, I examined whether clutch size differed when females were paired with heterospecific versus conspecific males, and whether conspecifics had higher fertilization success than heterospecific males.

### *Study System*

The spadefoot toads, *Spea multiplicata* (Mexican spadefoot toad), and *S. bombifrons* (plains spadefoot toad), co-occur across in the southwestern United States, including portions of Arizona, Texas and New Mexico (Stebbins, 2003). Where they co-occur, the two species breed in the same ponds thus may hybridize. Although *S. bombifrons*

and *S. multiplicata* are the most divergent species within *Spea* (Wiens and Titus, 1991), hybridization occurs in the wild (Forester, 1975; Simovich, 1985, Pfennig and Simovich, 2002). Hybridization rates are variable, but in some ponds it is greater than of 40% (Simovich, 1985; Pfennig and Simovich, 2002). Backcross individuals have also been found, suggesting introgression has occurred between these two species (Sattler, 1985). However, studies over the past thirty years suggest hybridization rates are declining, and that reinforcement may be occurring (Pfennig, 2003).

Reinforcement may be taking place between *S. bombifrons* and *S. multiplicata* in areas of sympatry, causing reproductive character displacement (Pfennig, 2000; Pfennig, 2003; Pfennig and Pfennig, 2005). Pre-mating barriers have therefore evolved between these two species. The existence of pre-zygotic barriers to fertilization has not been investigated. As mentioned previously, if females are sperm limited, they may benefit from hybridizing with a heterospecific male. Females of the two focal spadefoot toad species examined here potentially differ in whether they may obtain higher fertilization success when mating with a heterospecific male. Clutch sizes do not appear to differ between *S. bombifrons* (mean(SE) = 1160 (83) eggs) and *S. multiplicata* (mean(SE) = 1064 (46) eggs;  $t_{93} =$  ;  $p = 0.32$ ; K. Pfennig unpubl. data). However, males have differing fertilization rates, with *S. bombifrons* fertilizing 79% on average, and *S. multiplicata* fertilizing 90% on average (Wilcoxon normal approx.  $Z = -2.14$ ,  $p = 0.03$ ; K. Pfennig unpubl. data) when paired with a conspecific.

If species specific gamete receptors are not present, *S. bombifrons* females may gain higher fertilization success through mating with male *S. multiplicata*. Additionally, unless clutch size is reduced when mating with a heterospecific, *S. bombifrons* may gain a fitness advantage by mating with a heterospecific.

## Materials and Methods

To assess fertilization success in hybrids versus pure species, egg clutches were videotaped across four breedings occurring in the lab from 2005-2007. All pairs used in the breedings were from wild populations in Arizona and Texas. Only individuals collected from sympatric populations of *S. multiplicata* and *S. bombifrons* were used. All captured animals had been housed in a colony at the University of North Carolina at Chapel Hill for 1-5 years. All procedures were carried out in compliance with the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill.

Pairs were generated as 4 possible cross-types, 1) *S. multiplicata* (♀) x *S. multiplicata* (♂), 2) *S. bombifrons* (♀) x *S. bombifrons* (♂), 3) *S. multiplicata* (♀) x *S. bombifrons* (♂), and 4) *S. bombifrons* (♀) x *S. multiplicata* (♂). From this point on, these will be referred to as MM, BB, MB, and BM respectively, where the maternal parent is indicated first, followed by the paternal parent. Thus, two pure species genotypes (BB, MM) and two hybrid genotypes (MB, BM) were created. The fully crossed design allowed me to control for maternal effects.

To induce breeding in the lab, all toads were injected with 0.07mL of 0.1mM gonadotropin-releasing hormone. Each pair of toads was placed in a plastic nursery tank (42.4x27.9x17.5cm) filled with 9L of dechlorinated water and two 8-inch pieces of tubing. The tubing served as a possible attachment site for oviposition. Pairs remained in the nursery tanks overnight. Immediately following removal of adult pair, egg clutches were videotaped using a handheld video camera. Each nursery tank was placed over a grid containing 1in x 1in squares numbered from 1-120. Eggs were counted from recorded video frames. Fertilized eggs were determined by visualization of the animal pole (dark coloration on upper surface of eggs); unfertilized eggs appeared yellow in color, due to the presence of the vegetal pole at

the surface (Pfennig, 2000). For all clutches, eggs were assessed before the blastula stage, thus no biases occurred between fertilized, but unsuccessfully developed eggs. Development beyond the point of fertilization was not assessed.

### *Statistical Analysis*

All analyses were conducted using JMP 7.0.1. Clutch size was calculated as the total number of eggs a female laid in the nursery tank. Fertilization success was calculated as the number of fertilized eggs divided by total clutch size. To compare male fertilization success when paired with a heterospecific and conspecific mate, I performed pair-wise analyses for MM and MB, and then for, BB and BM. Fertilization success and clutch size were both non-normal, thus Wilcoxon tests were used. Additionally, I tested whether size of the clutch affected fertilization success. Populations from Arizona and Texas showed no differences between fertilization rate or clutch size, so analyses were performed using pooled data.

### **Results**

For *Spea multiplicata* females clutch size did not differ between conspecific (mean (SE) = 1249 (98), N= 22) and heterospecific males (mean (SE) = 921 (145), N= 10;  $Z = -1.545$ ,  $p = 0.11$ ; Figure 2.1). For *S. bombifrons*, females clutch size also did not differ when mated to conspecific (mean (SE) = 803 (127), N= 13) or heterospecific males (mean (SE) = 721 (139), N = 11;  $Z = -0.465$ ,  $p = 0.64$ ; Figure 2.2).

For *S. multiplicata*, males fertilization success did not differ with conspecific (mean (SE) = 97.01% (0.5%), N= 13) or heterospecific clutches (mean (SE) = 94.98% (1.6%), N = 11;  $Z = -0.671$ ,  $p = 0.49$ ; Figure 2.1). For *S. bombifrons*, males' fertilization success did not

differ with conspecific (mean (SE) = 97.4%(0.65%), N= 13) or heterospecific clutches (mean (SE) = 96.63%(0.86%), N = 11; Z= -0.637, p = 0.52; Figure 2.2).

Clutch size had no effect on overall fertilization success in any of the four groups: MM (F = 0.0122, p = 0.91); MB (F = 2.6659, p = 0.15); BB (F = 0.069, p = 0.80); BM (F = 0.312, p = 0.59).

## Discussion

Pre-zygotic barriers may arise between species that have been isolated and experienced divergence (Coyne and Orr, 2004). These barriers may play a key role in determining how populations interact upon secondary contact and whether hybridization will be selected against through reinforcement. My results suggest that species-specific gamete barriers are not present between *Spea multiplicata* and *S. bombifrons*. Both of my measures of pre-zygotic isolation, clutch size and fertilization success, did not differ between conspecific and heterospecific pairings. This is surprising since they are the most diverged species within *Spea* (Wiens and Titus, 1991). According to Sage et al. (1982) these two species have been diverged for 5-6 million years, thus we expected to see incompatibilities leading to lower fertilization success.

Although females of *S. bombifrons* and *S. multiplicata* do not appear to alter clutch size when paired with a conspecific or heterospecific male, female *S. multiplicata* do show a trend for larger clutch sizes when paired with a conspecific versus heterospecific male. In conspecific pairings, average clutch size is slightly over 1200 eggs; in heterospecific pairings, the average is slightly over 900 eggs. The lack of a significant result may be due to statistical power because twice as many conspecific pairs were observed. In conspecific



pairings, clutch sizes also show a much higher upper range from the mean (1100- 2200 eggs) than do females in heterospecific pairings (upper range = 1100-1300 eggs). Female *S. bombifrons* do not show a similar pattern. The upper ranges differ only by a few hundred eggs, while *S. multiplicata* differs by greater than a thousand.

Fertilization success for males in conspecific versus heterospecific pairings does not appear to differ for either *S. bombifrons* or *S. multiplicata*. Preliminary findings from K. Pfennig (unpubl. data) suggest that *S. multiplicata* males have a higher fertilization success than *S. bombifrons*. When paired with conspecifics, the two species produce clutches of similar size. *Spea multiplicata* females mating with an *S. bombifrons* male could be sperm limited and have a lower percentage of their clutch fertilized. I did not observe this pattern. *S. bombifrons* males had a 96-97% fertilization rate regardless of pairing. *S. multiplicata* had a very similar fertilization rate of 94-97%. My fertilization rates were thus higher than expected from previous results (K. Pfennig, unpubl. data), and may be an artifact of breeding in the lab. My results, nevertheless, indicate that females are not sperm limited and that males of both species produce ample sperm for fertilization.

While fertilization success did not differ between the pairings, *S. multiplicata* females have an interesting distribution of fertilization rates when paired with a heterospecific male that should be further explored in future studies. Clutches show a trend of either high success (>98%) or low fertilization success (<92%). None of the conspecific pairings had fertilization success below 92%, and the majority are centered around the mean of 97%. There appears to be a trend of having either high success or low success when paired with a male *S. bombifrons*. This may be indicative of divergence that has occurred between the two species when isolated; if the incompatible alleles have not yet reached fixation before secondary

contact, consequently, some males may be more or less incompatible than others. *Spea multiplicata* in southeastern areas of Arizona examined, have been exposed to *S. bombifrons* for a shorter time period than *S. bombifrons* has been to *S. multiplicata*. As *S. bombifrons* expanded its range southward, it made contact with *S. multiplicata* (Rice and Pfennig, 2008). Thus, *S. bombifrons* may have had more time to purge harmful alleles that could cause lower fertilization success when mated with a heterospecific. In the areas of Arizona where these toads were collected, there is evidence suggesting that secondary contact has only occurred in the last 100-150 years (Gerlbach, 1981; Bock and Bock, 2000), possibly explaining why maternal *S. multiplicata* hybrids show a pattern of low/high fertilization success. However, it is also possible that the low fertilization scores are a chance result of small males paired with large females. Further studies are needed to investigate the causes of this pattern.

Overall, my data do not support the presence of post-mating pre-zygotic barriers between these two spadefoot toad species. While pre-zygotic barriers do not appear, post-zygotic barriers are present; hybrids experience a continuum of reduced fecundity to complete sterility (Forester, 1975; Simovich, 1985). However, studies have also shown a fitness benefit of increased development time in maternal *S. bombifrons* tadpoles (Pfennig and Simovich, 2002), and future work could address whether this benefit is maintained over the course of post-metamorphic development and outweighs the fitness loss of impaired fecundity. Future work should also address the compatibility of male hybrid fertilization success in F2 and backcrosses to see if hybrid breakdown occurs (Burton, 1990; Li et al., 1997; Burton et al., 2006).

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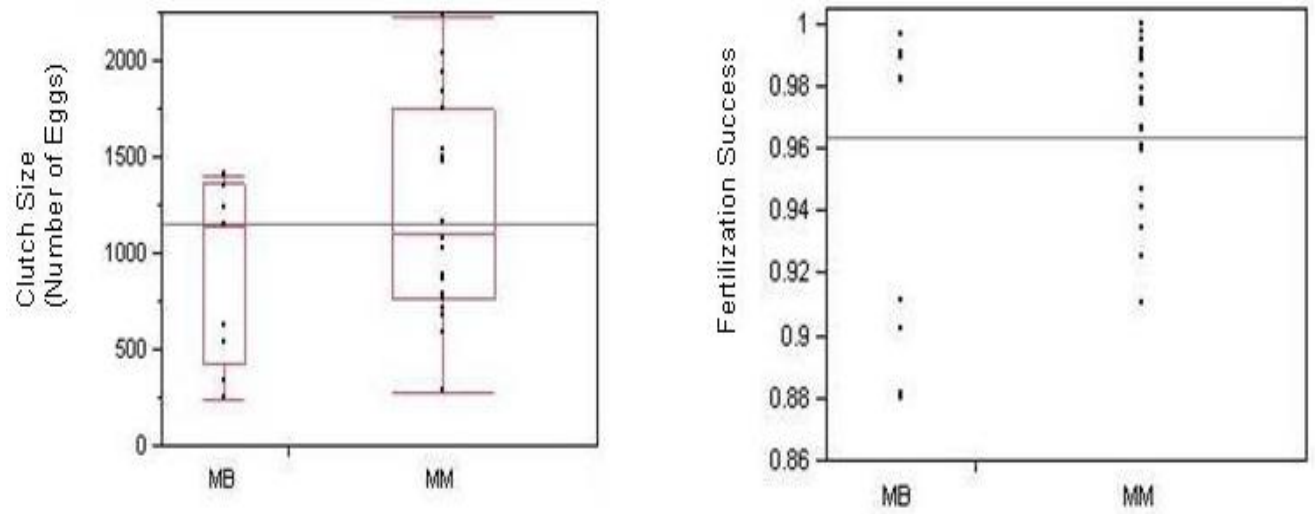
Wiens, J. J. and Titus, T.A. 1991. A phylogenetic analysis of *Spea* (Anura: Pelobatidae). *Herpetologica* 47:21–28.

## Figure Legends

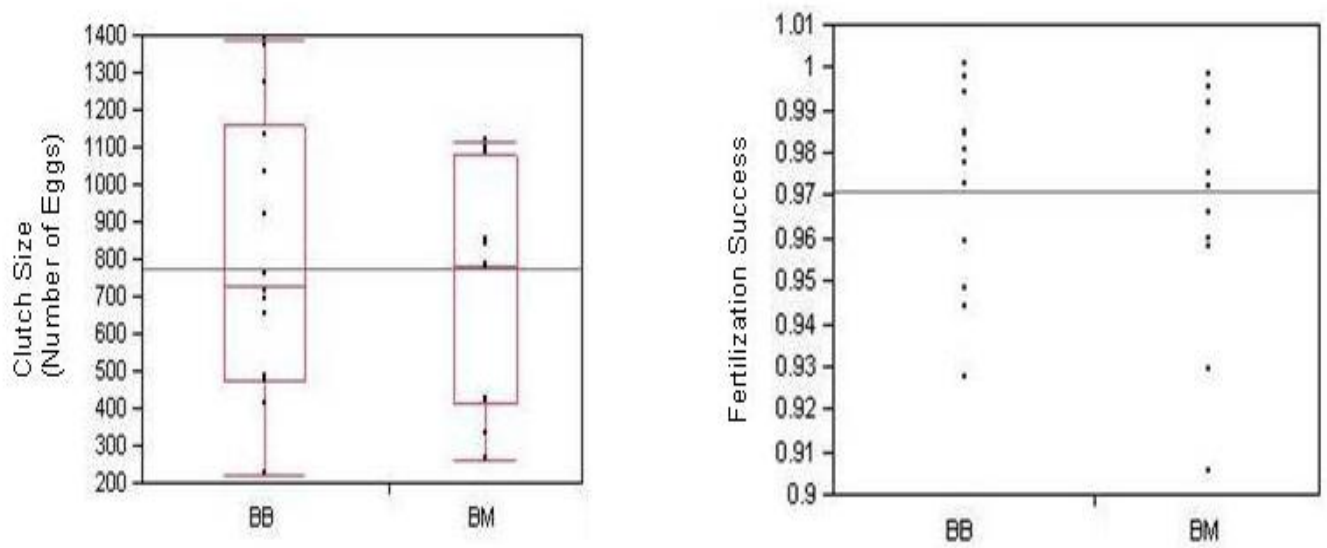
**Figure 2.1** A) Clutch size of *S. multiplicata* females paired with conspecific (MM) and heterospecific (MB) males. B) Fertilization success of males with conspecific (MM) and heterospecific (MB) clutches. No significant difference is present in either clutch size or fertilization success.

**Figure 2.2** A) Clutch size of *S. bombifrons* females paired with conspecific (BB) and heterospecific (BM) males. B) Fertilization success of males with conspecific (BB) and heterospecific (BM) clutches. No significant difference is present in either clutch size or fertilization success.

**Figure 2.1**



**Figure 2.2**



## CHAPTER 3

### **Post-metamorphic growth and survival in hybrids: are they as unfit as they seem?**

#### **Abstract**

The outcome of secondary contact depends on hybrid fitness; therefore, assessing hybrid fitness is crucial for predicting the evolution of reproductive barriers that prevent hybridization. Hybrid fitness is complex, however, and can vary depending on what components are being examined. I used two spadefoot toad species, *Spea bombifrons* and *S. multiplicata*, that have recently come into secondary contact, to examine the effects of hybridization on post-metamorphic growth and survival. Survival did not differ between hybrids and pure species offspring. Growth of hybrids was intermediate between parental phenotypes. Hybrids that have *S. bombifrons* as the maternal parent showed significantly impaired condition relative to other hybrids or pure species offspring. My data suggest that one species may benefit more than the other from hybridization.

#### **Introduction**

Hybrid fitness is a critical component that determines whether introgression between species is occurring. According to the biological species concept (Mayr, 1942), reproductive isolation characterizes species. Low hybrid fitness is a hallmark of “good species” and as such is often used to delineate species populations (Coyne and Orr, 2004). The costs of hybridization at one life stage may be severe, but favored during another (Parris et al., 1999;



Parris, 1999). Further complicating hybridization studies is the possibility that the hybridizing species may differ in the strength of selection against hybridization (Parris et al., 1999; Pearson, 2000; Tiffin et al., 2001; Veen et al., 2001; Pfennig and Simovich, 2002). One way in which this might occur is if hybrids perform intermediate relative to parental species. Selection may favor one species, but not the other in these situations (Parris et al., Pearson, 2000; Pfennig and Simovich, 2002). When selection favors one species, this may impede reinforcement (Kelly and Noor, 1996; Servedio and Kirkpatrick, 1997) and introgression of genes between the two species can occur.

Because hybridization is so complex, several components of hybrid fitness need to be examined to fully understand how populations coming into secondary contact will interact. If hybrids are unfit, selection may favor pre-mating isolating barriers, which will eventually end hybridization between the populations (Howard, 1993; Coyne and Orr, 2004). However, hybrids are not always unfit (Arnold, 1997). Hybrids can be as fit as parents, in which case the species will continue to interbreed and a stable hybrid zone may form, or they can have higher fitness relative to parents, in which case they may become reproductively isolated and form a new species (Arnold, 1997). Assessment of the costs and benefits of hybridization for both species and across multiple life stages is necessary to gain an accurate picture of hybrid fitness.

Amphibians have a complex life-cycle that allows measurement of fitness across several life-history stages, each of which is critical to total fitness. Using two species of spadefoot toads, *Spea bombifrons* and *S. multiplicata*, I examined growth and survival in early post-metamorphic hybrid toads. The study system is outlined below.

### *Study System*

The spadefoot toads, *Spea multiplicata* (Mexican spadefoot toad), and *S. bombifrons* (plains spadefoot toad), co-occur across in the southwestern United States, including portions of Arizona, Texas and New Mexico (Stebbins, 2003). Where they co-occur, the two species breed in the same ponds and may hybridize. Although *S. bombifrons* and *S. multiplicata* are the most divergent species within *Spea* (Wiens and Titus, 1991), hybridization occurs in the wild (Forester, 1975; Simovich, 1985; Pfennig and Simovich, 2002). Hybridization rates are variable, but in some ponds it is greater than 40% (Simovich, 1985; Pfennig and Simovich, 2002). Backcross individuals have also been found, suggesting introgression has occurred between these two species (Sattler, 1985). However, studies over the past thirty years suggest hybridization rates are declining, and that reinforcement may be occurring (Pfennig, 2003).

Hybrid fitness is severely reduced in adult hybrids (Forester, 1975; Simovich, 1985). Simovich (1985) found that females are partially fecund, with half as many eggs as pure species types. Males are thought to range from only partially (Forester, 1975) to completely sterile (Simovich, 1985). Even in light of this reduced adult fitness, for *Spea bombifrons* females it may be advantageous in low water conditions to hybridize with *S. multiplicata* males to attain faster development time in tadpoles (Pfennig, 2008). Pond disappearance time may be a strong selective force in some populations. In one study of *S. multiplicata*, conducted by Pfennig (1990), 11% of surveyed ponds dried before any tadpoles were able to metamorphose. In another study conducted by Newman (1987), desiccation was the major cause of larval mortality in *Scaphiopus couchii*, another spadefoot toad that occurs in the southwestern United States. However, faster development time can mean smaller size. There exists a tradeoff between metamorphosing early at a small size to avoid predation and

possible drying of a pond and metamorphosing later at a larger size which could increase survival outside of the pond (Pfennig et al., 1991; Werner, 1986). The advantage of early emergence from the pond for *S. bombifrons* may be balanced by compromised survival following metamorphosis.

In the wild, metamorphosis usually occurs between 27-28 days post fertilization for *S. bombifrons* and 24-25 days for *S. multiplicata* (Pfennig and Simovich, 2002). Following metamorphosis, juveniles usually spend a few weeks above ground foraging for food (Bragg, 1965). Between 4-6 weeks post metamorphosis, the juveniles burrow underground. They remain underground for almost an entire year, emerging in the late spring/early summer with the coming of rain to forage again (Bragg, 1965). In nature, reproductive maturity is not reached until two to three years post metamorphosis. Little is known about hybrid growth and survival from metamorphosis to adult.

While several studies have focused on hybridization in spadefoot toads, they have examined only two life-stages: early development prior to metamorphosis and adult. The period immediately following metamorphosis is critical in many amphibian species because it is a time of rapid growth and body restructuring. In *Spea* it is a critical time of growth, representing the only window of foraging time and growth that occurs before the next breeding season (Bragg, 1965; Tinsley, 1990).

## **Materials and Methods**

To study the effects of hybridization in two spadefoot toads species, I crossed *Spea multiplicata* and *Spea bombifrons*. These two species co-occur in several areas of the southwestern United States and naturally hybridize (Forester, 1975; Simovich, 1985; Pfennig

and Simovich, 2002). All pairs used in the breedings were captured near Portal, Arizona. Only individuals collected from sympatric populations of *S. multiplicata* and *S. mbombifrons* were used. All captured animals had been housed in a colony at the University of North Carolina at Chapel Hill for 1-5 years. All procedures were carried out in compliance with the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill.

I created four cross-types, where I bred *S. multiplicata* (♀) x *S. multiplicata* (♂), *S. bombifrons* (♀) x *S. bombifrons* (♂), *S. multiplicata* (♀) x *S. bombifrons* (♂), and *S. bombifrons* (♀) x *S. multiplicata* (♂). From this point on, these will be referred to as MM, BB, MB, and BM respectively, where the maternal parent is indicated first, followed by the paternal parent. I conducted two breedings, one in the spring (May 2008), and a second in the fall (October 2008). In the May breeding, I bred 5 pairs of MM, 7 pairs of BB, 5 pairs of MB, and 5 pairs of BM. Of these, we had 2 MM pairs, 2 BB pairs, 2 MB pairs, and 5 BM pairs produce eggs that developed into tadpoles. In the October breeding, I bred 4 pairs of MM, 6 pairs of BB, 6 pairs of MB and 6 pairs of BM. Of these, I had 3 MM pairs, 4 BB pairs, 4 MB pairs, and 2 BM pairs produce eggs that developed into tadpoles. To induce breeding in the lab, all toads were injected with 0.07mL of 0.1mM gonadatropin-releasing hormone. Each pair of toads was placed in a plastic nursery tank (42.4x27.9x17.5cm) filled with 9L of dechlorinated water and two 8-inch pieces of tubing. The tubing served as a possible attachment site for oviposition. The pairs remained in the nursery tanks overnight and were removed the following morning. All tanks containing eggs were equipped with aerators. Three days following the breeding, tadpoles started to swim freely in the tanks and were fed twice daily with tadpole food. Aeration was discontinued at this time. In the May breeding,

tadpoles were fed Nasco frog brittle. Due to a high mortality rate following this breeding, I switched to Carolina Biological *Xenopus* Tadpole food for the October breeding. Tadpoles were removed from these nurseries at Gosner stage 42 (marked by appearance of forelimbs) and placed in a beach tank containing water and sand, providing dry areas for the tadpoles. At Gosner stage 46 (marked by complete tail absorption), tadpoles had fully metamorphosed into toadlets and were moved into adult boxes. (14x14x21.5cm). Bedding material differed for the May and October breedings. In May, the toadlets were housed on moist sand identical to the adult tanks. However, due to high mortality of juveniles in this breeding, I switched to moist coconut core for the October breeding. In both breedings, bedding material was kept moist by weekly watering. All juveniles were fed several times per week, on a diet consisting of crickets dusted with nutrients. All juveniles were kept at an initial density of 4 individuals/box. These individuals reached Gosner stage 46 at approximately the same date. If an individual died, it was not replaced, thus density changed over the length of the experiment. Within a box, each toadlet was individually marked with a visible implant elastomer (Northwest Marine Technology, Inc.). Snout vent length (SVL) and mass were taken every week for a period 6 weeks for each individual. Additionally, any mortality was recorded to determine survivorship of hybrids (BM, MB) versus pure species (MM, BB) offspring.

### *Statistical Analysis*

To meet the assumptions of parametric tests (i.e. normal distribution), SVL and mass were log transformed. Raw mass was highly correlated with SVL, thus to control for this, I computed the residuals of a cubic regression of mass (log transformed) on SVL (log

transformed). This gave the overall “condition” of the toadlets. All analyses from here on out were carried out on “condition” rather than raw mass.

Age to metamorphosis was calculated based on the first sixteen tadpoles to completely metamorphose for each sibship. Nursery tanks were not depleted of water over time, thus creating an artificially long-lasting environment for growth. I only included tadpoles in my analysis that reached metamorphosis by 60 days post breeding. Due to this cut off, which represents natural duration in the field, not all sibships included in the analysis had sixteen tadpoles. My analyses include 4 sibships of BB (two of which did not have sixteen tadpoles develop before day 60, so only 7 tadpoles are represented by these sibships); 2 sibships of BM, 4 sibships of MB, and 3 sibships of MM (two of which did not have sixteen tadpoles develop before day 60, so only 14 and 9 tadpoles are represented by these sibships). I used a Wilcoxon test, and Tukey post hoc tests to make pair-wise comparisons.

Because individuals within a box were not independent, SVL and mass were averaged across all individuals to obtain a single measure per box, except in analyses involving condition at metamorphosis. Each measure in these analyses represents a single toad. Survival was calculated as the number of individuals per box remaining after 6 weeks. All bivariate fits and ANOVAs were conducted using JMP 7.0.1.

Due to the overwhelming mortality experienced in the May breeding, survival analysis was only conducted on the October breeding data.

## **Results**

Age at metamorphosis differed significantly among the four groups (ChiSquare = 31.2532,  $df=3$ ,  $p = 0.0001$ , Figure 3.1). Pair-wise comparisons using a Tukey Post hoc test

revealed that BB, MM, and MB had no difference, but that BM had a significantly faster development time than all three. Average age at metamorphosis was: BB 46 days, BM 39 days, MB 45 days, and MM 44 days (Figure 3.1). These data reflect only the October breeding.

As expected from previous studies (Pfennig and Simovich, 2002), BB had the highest survival 6 weeks post metamorphosis. MM had the lowest survival. However, there was no significant difference in survival among the four groups (ChiSquare 1.5495,  $df = 3$ ,  $p = 0.67$ , Table 3.1).

Condition did not predict survival probability for any of the genotypes (Table 3.2). The condition of the juveniles that died was not different from those that survived 6 weeks post-metamorphosis.

Survival decreased with age to metamorphosis for BM, and MM; it is marginally significant for MB (Table 3.3, Figure 3.2). BB showed no difference in survival among tadpoles that metamorphosed early or late (Table 3.3, Figure 3.2).

Condition and age at metamorphosis are correlated in the literature, with later developing tadpoles having higher condition (Semlitsch and Scott, 1988). I, therefore, examined the relationship between age at metamorphosis and condition at metamorphosis. Condition does not differ between tadpoles metamorphosing early or late for BB ( $R^2 = 0.002$ ,  $F = 0.0753$ ,  $p = 0.7853$ ,  $df = 39$ ), BM ( $R^2 = 0.031$ ,  $F = 2.1397$ ,  $p = 0.01483$ ,  $df = 38$ ), or MM ( $R^2 = 0.0123$ ,  $F = 0.8095$ ,  $p = 0.3716$ ,  $df = 66$ ; Figure 3.3). However, condition increased with later age in MB ( $R^2 = 0.049$ ,  $F = 5.2089$ ,  $p = 0.0245$ ,  $df = 103$ ).

Breeding date had a significant effect on size and condition ( $F = 111.476$ ,  $p = 0.0001$ ,  $df = 1$ ), therefore, the two analyses were examined separately. Hybrids show no consistent

differences in growth over time relative to pure species types. Initially, in both breedings, hybrids have intermediate SVL and mass compared to pure species offspring, (Figure 3.4). In the October breeding, MB hybrids also show intermediate condition and BM hybrids show lower condition relative to pure species types; however, in May, hybrids show decreased condition relative to pure species types (Figure 3.4). With time, the October breeding shows BM with a slower growth rate relative to pure species types in both (SVL) and mass, conveying the lowest condition for weeks two through six.

## **Discussion**

Isolated populations can diverge due to mutation, drift and selection (Coyne and Orr, 2004). Upon secondary contact, populations may have diverged sufficiently so that hybridization does not occur. However, if hybridization does occur between the incipient species, the divergence could cause post-zygotic barriers including hybrid inviability or sterility (Coyne and Orr, 2004). In the spadefoot toads, *Spea bombifrons* and *S. multiplicata*, hybridization may confer a fitness advantage in early development, but a fitness decrease in reproduction once sexual maturity is reached (Pfennig and Simovich, 2002). How hybrids fare in the interim-after metamorphosis, but before reaching reproductive age- could influence whether hybridization is selected for or against in this system. This has implications for how speciation progresses.

In amphibians, slow development is often correlated with larger size at metamorphosis (Wilbur and Collins, 1973; Wilbur, 1980). Larger size at metamorphosis may provide greater survival post metamorphosis (Wilbur, 1980), although several studies have demonstrated that size at metamorphosis has no effect on survival (Berven, 1990; Semlitsch



et al., 1988). However, in natural populations of *S. multiplicata* an inverse relationship has been found; body size decreases with increased time to metamorphosis (Pfennig et al., 1991). Pfennig hypothesized this was a result of decreasing food over time in the ponds. An additional hypothesis is that when tadpoles are not food limited, patterns and size at metamorphosis reflect natural variation in growth among individuals and thus, some individuals develop sooner at a larger size. Food was a limiting factor in the Pfennig et al. study (1991), so the natural variation hypothesis was rejected. Berven (1990) found a similar pattern in *Rana sylvatica*, but could not explicitly base it on limited food resources. My results on body size at metamorphosis are somewhat surprising since the tadpoles were raised in ideal conditions with low density and high food levels throughout development. An inverse relationship did not occur because of diminishing resources within our tadpole tanks. Juveniles that metamorphose at an early age are strongly selected for due to the brevity of pond endurance. These quickly developing tadpoles may represent variation within the population which includes some high quality individuals developing quickly and at a large size. My results may only be a snapshot into genetic variation within a population and not a summation of the entire whole. Future research should focus on examining this relationship with more adult pairs.

The differences observed in growth and survival between the May and October breedings may illustrate how hybrids respond to different environmental conditions. Again, while the results in growth and survival did not differ between groups, patterns have emerged. The May breeding may have represented a “harsh” environment where food was limited and the substrate too heavy for burrowing by the young juveniles to burrow into and out of. Mortality exceeded 95% in May. In October, food resources were more plentiful and

the substrate was less dense, possibly creating a more suitable or “ideal” environment. Mortality rate was less than 30%. Hybrids may perform more poorly in “harsh” conditions relative to pure species types; represented by the pattern of low condition relative to parents. In “ideal” conditions, MB hybrids performed intermediate to pure species, but BM had lower condition. Intrinsic incompatibilities may become more apparent in “harsh” or stressful environments. BM may also have a higher level of intrinsic incompatibilities that are apparent regardless of environmental condition. Hybridization between *S. bombifrons* females and male *S. multiplicata* is the most common pairing observed in nature (Pfennig and Simovich, 2002), thus selection on BM hybrids may be greater than on MB hybrids. Comparing “harsh” and “ideal” conditions was not a focus of this study, rather a by-product; as such, further testing should be done to examine the role of environmental conditions in hybrids and pure species.

Experimental evidence has shown that BM tadpoles metamorphose at a rate 4-5 times faster than pure *S. bombifrons* tadpoles (Pfennig and Simovich, 2002). In this case, even when males are sterile and females half as fecund as pure species offspring, hybridization may confer a fitness advantage in ephemeral ponds. In this study, however, I found that the overall condition of BM juveniles is lower than BB juveniles. Future research should examine this relationship with greater sibship numbers. The pattern of decreased condition was consistent across both the October and May breedings. If the juvenile BM toads suffer poor condition following metamorphosis, they may suffer increased mortality relative to BB offspring. Hybridization may be disfavored under these conditions.

My results show that even though there are circumstances where hybridization could benefit *S. bombifrons* females regardless of loss in fertility at maturity, mortality rate is

potentially increased in the early juvenile stage. Although BM did not show a significantly higher death rate relative to BB, the study was conducted laboratory and not in field conditions which can be harsh and unyielding at times. Hence, selection pressure to avoid hybridization may be stronger in this system than previously thought.

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## Figure Legends

**Figure 3.1.** Days to metamorphosis for hybrids and pure species. (Average based on first 16 tadpoles to metamorphose for each sibship within each treatment group. Error bars represent 95% confidence interval. BB represented by 4 sibs, MM by 3, MB by 4, and BM by 2.) Data from October breeding only.

**Figure 3.2** Relationship between age at metamorphosis and likelihood of survival at six weeks post-metamorphosis. Dots represent average survival across each replicate. Each replicate was an average of 4 toads housed within a single box. All genotypes show a significant increase in mortality with increased age, except for BB.

**Figure 3.3.** Relationship between age at metamorphosis and condition at metamorphosis. There was no significant increase in condition with increased age for all groups with the exception of MB. MB showed improved condition with increasing age to metamorphosis.

**Figure 3.4.** Growth and condition curves over six weeks following metamorphosis. Graphs A-C represent data from the October breeding; D-F represent growth patterns from the May breeding. Errors bars are 95% confidence intervals. Graphs A and D are of log transformed SVL. B and E are log transformed mass. Finally, graphs C and F show condition.

Figure 3.1

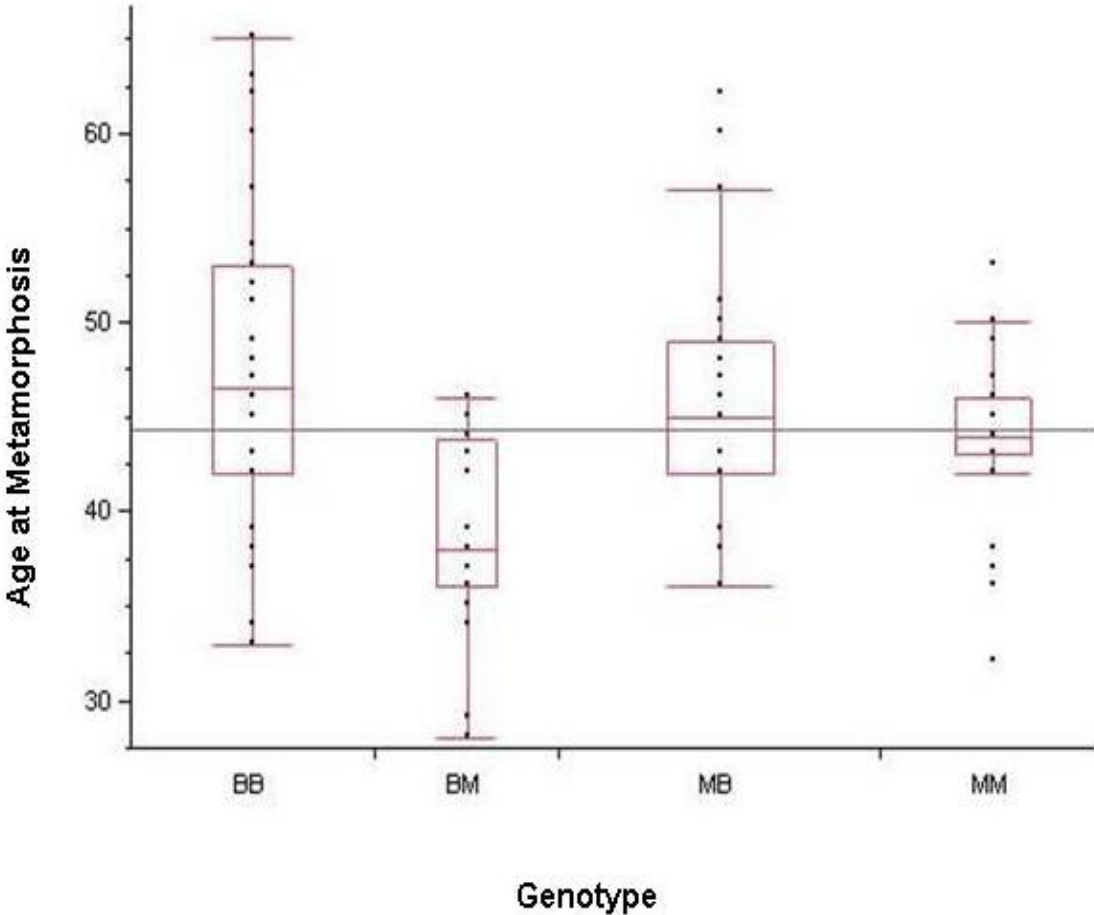




Figure 3.2

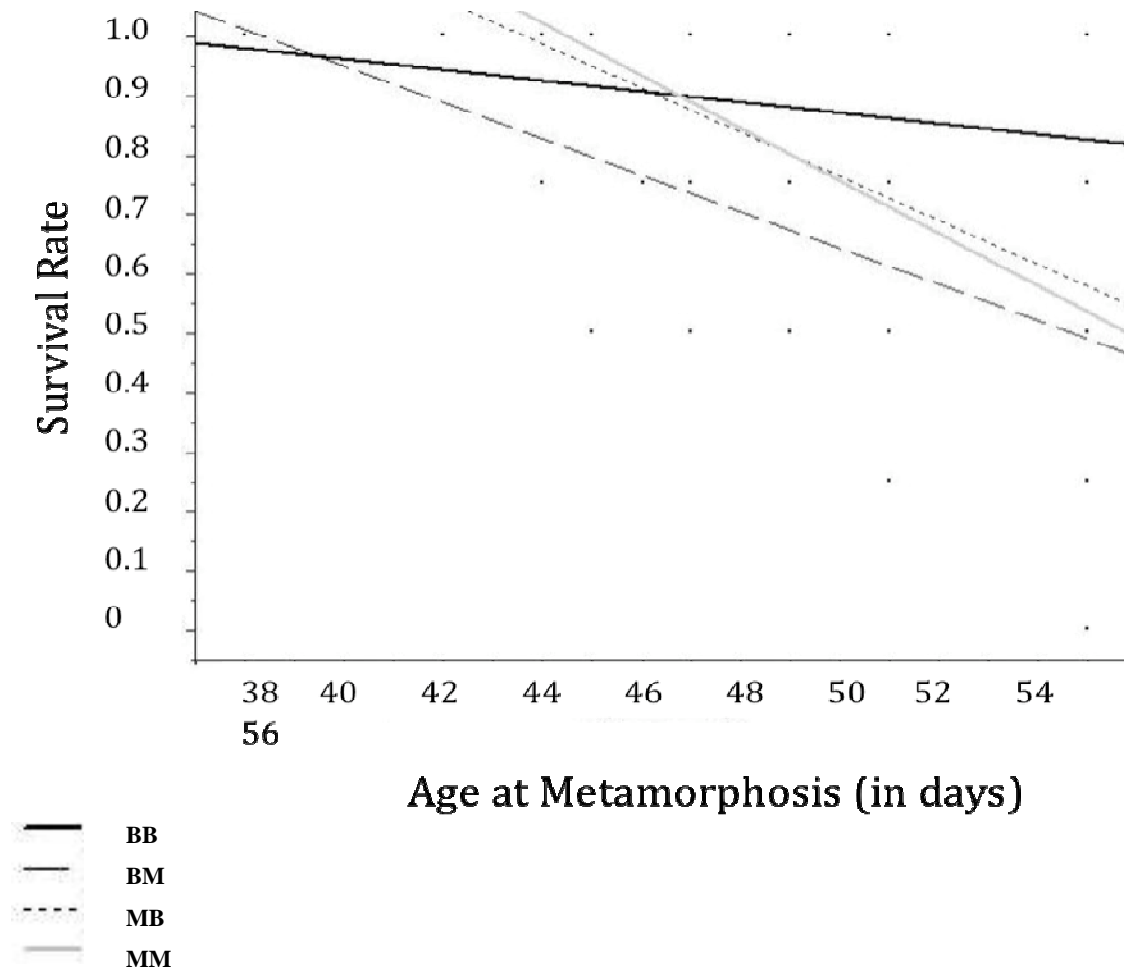
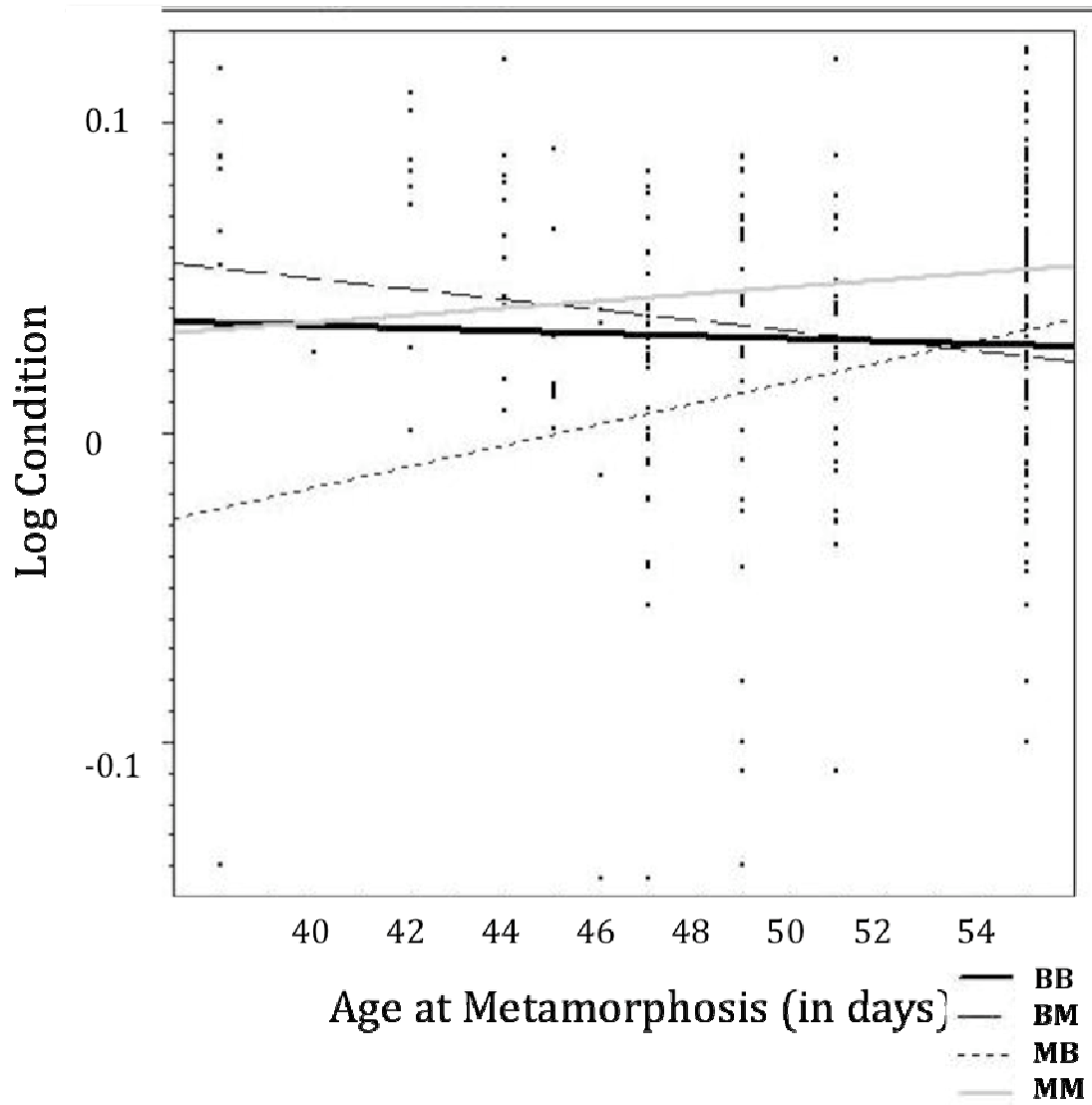
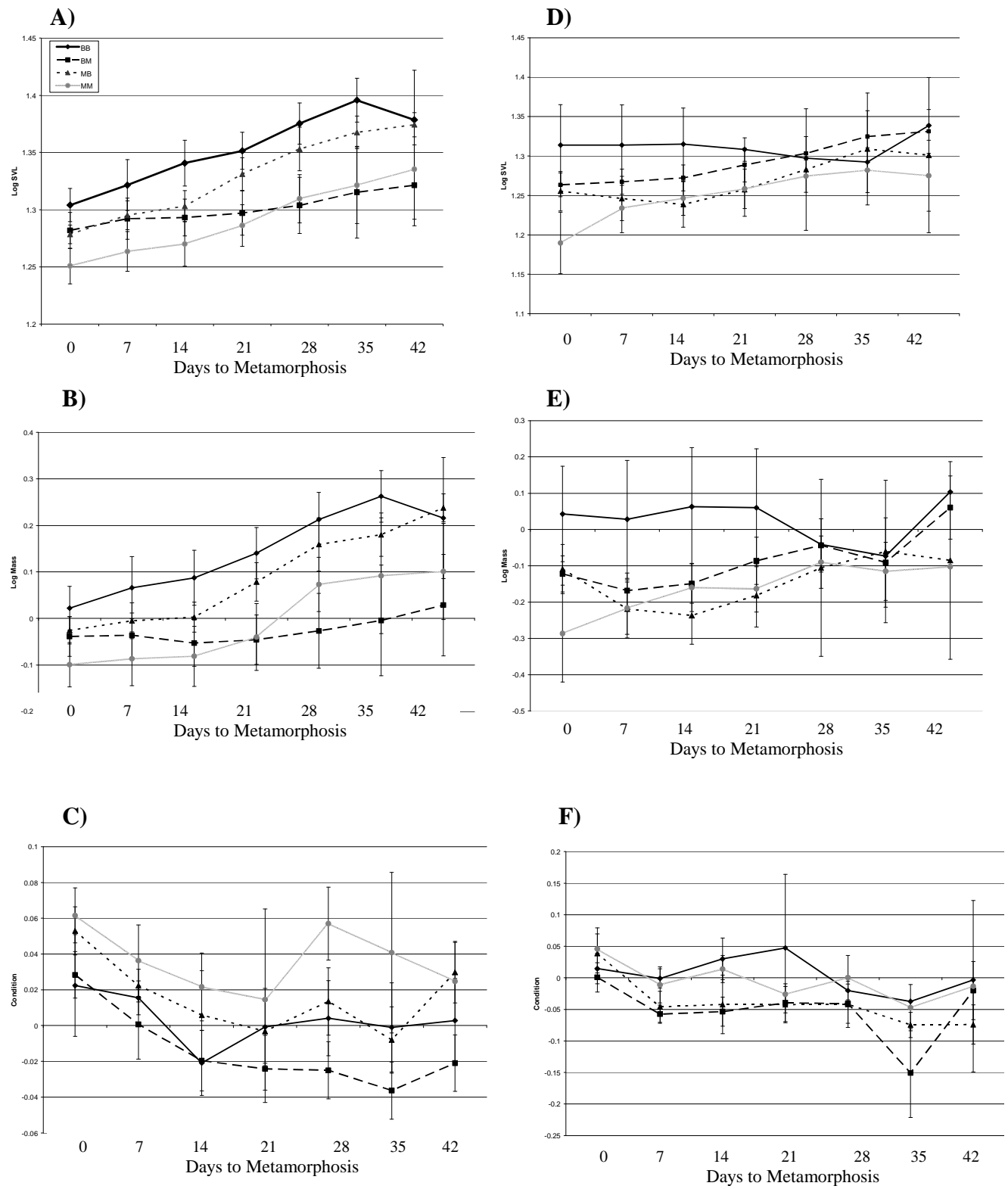


Figure 3.3



**Figure 3.4**



**Table 3.1.** Probability of survival 6 weeks post metamorphosis.

Genotype	BB	MM	BM	MB
Probability of Survival	0.75	.618	.700	.667

**Table 3.2.** Summary statistics for condition as a predictor of survival six weeks post-metamorphosis. For all groups degrees of freedom = 1.

Genotype	BB	BM	MB	MM
ChiSquare	0.1429	0.024	0.180	0.086
P value	0.701	0.878	0.665	0.787

**Table 3.3.** Statistics summary for survival as a factor of days post breeding.

Genotype	BB	BM	MB	MM
RSquare	0.0877	0.408	0.1472	0.2640
DF	9	14	25	17
P value	0.4059	0.0103	0.0530	0.029

## CHAPTER 4

### Conclusions

Although hybridization is crucial to the processes of speciation, very few studies have taken into account the complexity of hybridization. Hybrid fitness can vary depending on the fitness components measured, life history stage examined, and the environmental context (Pearson, 2000; Parris 2001). Additionally, the strength of selection to avoid hybridization may differ for each of the hybridizing species (Pearson and Rohwer, 1998; Tiffin et al., 2001; Pfennig and Simovich, 2002).

Total hybrid fitness can not be assessed through measurement of a single fitness component. Hybridization is a complex process that must be examined in multiple contexts in order to understand how it can facilitate or impede reproductive isolation between populations. There may be fitness effects for females from hybridization immediately upon mating. The number of offspring a female produces is directly tied to fertilization success, and this may be improved or impaired with a heterospecific male. Hybrid fitness following fertilization becomes contingent on the offspring's ability to: grow and develop normally; survive to reproductive maturity, produce viable gametes; and mate. Evaluating each of these components is thus necessary to understand hybrid fitness and how hybridization affects the focal populations.

Using spadefoot toads as my system, I examined both pre and post-zygotic fitness components in hybrids. *Spea bombifrons* and *S. multiplicata* co-occur in areas of the

southwestern United States, and naturally hybridize in areas of sympatry (Pfennig and Simovich, 2002). Previous studies of pre-metamorphic fitness in hybrids found that hybrids where *S. bombifrons* is maternal develop more quickly than pure species *S. bombifrons* offspring (Pfennig and Simovich, 2002). In adult hybrids, however, females are partially fecund and males exhibit a range of sterility from complete to only partially sterile (Forester, 1975; Simovich, 1985). The above studies suggest that selection could favor hybridization in *S. bombifrons* if the fitness advantage gained in development is larger than the cost of lost fertility as an adult. My study was designed to analyze two missing components: the fitness effects of mating with a heterospecific, and the fitness of hybrids in post-metamorphic growth before reaching maturity.

First, I found that no pre-zygotic barriers to hybridization are present between *S. bombifrons* and *S. multiplicata*. Fertilization success did not differ between heterospecific and conspecific males. Additionally, clutch size did not differ between females paired with heterospecific or conspecific males. These findings suggest that species specific gamete recognition is not present in this system. At the pre-zygotic level, hybridization neither promotes nor deters reproductive isolation from occurring.

Second, I found that in the six week period immediately following metamorphosis, hybrids did not have higher mortality rates than either *S. bombifrons* or *S. multiplicata* offspring. Growth and condition did not differ significantly from pure species types, although a pattern of low condition was found for BM hybrids. Tadpoles metamorphosing the earliest

had the highest chance of survival. Condition at metamorphosis did not differ with age, and was not a predictor of survival six weeks post-metamorphosis.

In conclusion, hybrid fitness is complex, and varies across different life stages and with different fitness components. It appears that hybridization between *S. bombifrons* and *S. multiplicata* is moderated by pre-mating barriers and post-zygotic fitness costs accrued at reproductive maturity. Growth and survival may be impaired in the early post-metamorphic period, but my results are not conclusive. Further research should focus on examining early post-metamorphic growth and survival in natural conditions.

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