ECOLOGICAL CHARACTER DISPLACEMENT AND ITS CONSEQUENCES: POPULATION GENETIC ANALYSES IN SPADEFOOT TOADS

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ABSTRACT

AMBER MARIE RICE: Ecological Character Displacement And Its Consequences: Population Genetic Analyses In Spadefoot Toads
(Under the direction of David W. Pfennig)

Ecological character displacement, or trait evolution stemming from resource competition, occurs when selection to avoid resource competition favors individuals of two competing species who are least like the other species in resource use traits. Character displacement is an important mechanism driving adaptive radiation and species coexistence, and it has been documented in many taxa. Yet, many factors that affect the evolution of character displacement and its consequences remain unclear. My dissertation research seeks to address this gap.

Character displacement may evolve through two non-exclusive routes that differ in the source of phenotypic variation, and hence, in the ease with which character displacement unfolds. I discuss differences between these routes, review possible examples of each, and describe how distinguishing between them provides insight into factors that affect the evolution of character displacement and its possible consequences.

When resources are asymmetric, character displacement may lead to differential fitness consequences between competing species, creating a "winner" and a "loser." Using population genetics, I established that the winner in a case study of character displacement—spadefoot toads—was the more recent invader into the region where

character displacement has occurred. I suggest that because superior competitive abilities may facilitate invasions, invaders may generally win during character displacement.

In putative cases of character displacement, it is important to establish that selection, and not chance, has been primarily responsible for generating trait divergence. One way to do this is to demonstrate that multiple populations have diverged independently. Using a population genetics approach, I found that multiple sympatric populations of the spadefoot toad *Spea multiplicata* have independently diverged from allopatric populations. In addition to supporting the role of selection in this case of character displacement, my results also clarify by which route this species underwent character displacement.

Finally, an indirect consequence of character displacement is that it may initiate speciation between conspecific populations experiencing different competitive environments. With genetic data, I found evidence of a slight reduction in gene flow between *S. multiplicata* populations in different competitive environments. These data therefore support the suggestion that speciation may arise as an indirect consequence of character displacement.

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CHAPTER I

GENERAL INTRODUCTION

"Natural selection, also, leads to divergence of character; for more living beings can be supported on the same area the more they diverge in structure, habits, and constitution...and during the incessant struggle of all species to increase in numbers, the more diversified these descendants become, the better will be their chance of succeeding in the battle of life" (Darwin 1859). With these words from the *Origin of Species*, Charles Darwin described a process he called divergence of character, in which individuals more different from their competitors experience higher fitness. This process explained the presence of a common pattern in nature: that closely related species are often recognizably different where they occur together versus where they occur separately. Since that time, the process of divergence of character has become known as character displacement (Brown & Wilson 1956), and has been studied in many taxa (reviewed in Howard 1993; Schluter 2000b; Dayan & Simberloff 2005).

Character displacement, or interspecific trait divergence stemming from selection to avoid resource competition or interspecific interactions during mating, has long been recognized as an important mechanism of species divergence, coexistence, and adaptive radiation (reviewed in Howard 1993; Schluter 2000b; Coyne & Orr 2004; Day & Young 2004). This process can occur in two forms: reproductive character displacement, where selection to avoid interspecific interactions during mating leads to divergence between

species in mating characters (i.e., male signals and/or female preferences; reviewed in Howard 1993; Coyne & Orr 2004); and ecological character displacement, where selection to avoid interspecific resource competition leads to divergence between species in resource use and associated phenotypes (reviewed in Robinson & Wilson 1994; Schluter 2000b; Day & Young 2004; Dayan & Simberloff 2005). In this dissertation, I focus mainly on ecological character displacement. However, because the same mechanism—selection to minimize interspecific interactions—drives these two processes, many of the conclusions may be applicable to both forms of character displacement.

The pattern of greater divergence between species in sympatry versus allopatry may be explained by mechanisms other than interspecific competition in sympatry (Grant 1972; Arthur 1982). Because of this, much research has been devoted to gathering evidence (Schluter & McPhail 1992; Taper & Case 1992) to document the process of ecological character displacement in many systems (reviewed in Schluter 2000b). Yet, many of the factors that affect the evolution of character displacement and its consequences remain unclear. In Chapter II, I describe two non-mutually-exclusive routes by which character displacement may evolve—*in situ* evolution of novel phenotypes and sorting of pre-existing variation. I discuss how distinguishing between these two routes may help to explain the speed of character displacement, predict the likelihood of character displacement triggering further diversification, and understand the ultimate origins of divergent phenotypes. This chapter has been modified from Rice, A. M. and Pfennig, D. W. 2007. Character displacement: *in situ* evolution of novel phenotypes or sorting of pre-existing variation? *Journal of Evolutionary Biology* **20**: 448-459.

When resources are asymmetric in quality, the species that monopolizes the better resource after character displacement may have relatively higher fitness, and therefore be considered the "winner," compared to the species that monopolizes the lower quality resource (the "loser"; Pfennig & Pfennig 2005). Two factors that may affect the likelihood of a particular species to monopolize the higher quality resource are whether that species is a more recent invader into the region where character displacement is taking place, and if it is, historical selection in the ancestral range. In Chapter III, I use population genetics to investigate these factors in a case study of character displacement—spadefoot toads. Chapter III has been modified from Rice, A. M. and Pfennig, D. W. 2008. Analysis of range expansion in two species undergoing character displacement: why might invaders generally 'win' during character displacement? *Journal of Evolutionary Biology* **21**: 696-704.

When documenting a putative case of character displacement, it is necessary to rule out chance as an explanation for the pattern of divergent traits in sympatry. This is often done by measuring phenotypes in multiple sympatric and allopatric populations, and demonstrating that divergence is greater than expected by chance. In most studies, however, no evidence is presented to establish the independence of these populations (Schluter 2000a). Thus, even when multiple conspecific populations in sympatry exhibit a phenotype that has diverged from those in allopatry, it remains possible that the divergent phenotype only evolved once, and then subsequently spread to other sympatric populations via gene flow. In such a case, character displacement has not been replicated, and the evidence against chance is weakened. To distinguish between this scenario and one in which divergence has occurred multiple times independently, it is necessary to

combine genetic data with morphological and ecological data. In Chapter IV, I use population genetics and morphology to ask whether phenotypic divergence has occurred repeatedly in independent populations of a spadefoot toad species undergoing character displacement.

Finally, in Chapter V, I turn to one potential by-product of character displacement—the evolution of reproductive isolation between conspecific populations in sympatry and allopatry with a competitor (Pfennig & Rice 2007; Rice & Pfennig 2007). Traditionally, character displacement has been considered a mechanism by which the process of speciation may be finalized. Often overlooked, however, is the potential for character displacement to initiate speciation. Conspecific populations in sympatry and allopatry with a competitor experience different environments. Local adaptation to these divergent environments may lead to the evolution of reproductive isolation (Pfennig & Rice 2007). If such isolation is present, the amount of gene flow between sites in allopatry and sympatry should be reduced relative to the amount of gene flow among sites within each of these regions. In Chapter V, I used population genetics to test for such a reduction in gene flow between sympatric and allopatric breeding aggregations of a spadefoot toad species.

Spadefoot Toads

In Chapters III, IV, and V, I investigated ecological character displacement and its consequences in the Mexican and Plains spadefoot toads (*Spea multiplicata* and *S. bombifrons*, respectively). The ranges of these species overlap in the southwestern United States (Stebbins 2003), creating the potential for local co-occurrence. *Spea* are adapted to

living in arid, desert environments. They spend much of the year in underground burrows, emerging during the summer rainy season to feed and to breed (Bragg 1944, 1945). Spadefoots breed on the evening following a rainstorm, in ephemeral ponds formed by run-off (Bragg 1945).

Spea tadpoles exhibit developmental polyphenism. They can develop as either a small-headed omnivore morph, which feeds on organic detritus at the bottom of the pond, or as a large-headed carnivore morph, which specializes on anostracan fairy shrimp in the water column (Pomeroy 1981; Pfennig 1990). Carnivores have enlarged jaw muscles (the orbitohyoideus (OH) and interhyoideus (IH) muscles) and serrated beaks (Pomeroy 1981; Pfennig 1992b), which likely improve foraging ability on fairy shrimp (Satel & Wassersug 1981; Ruibal & Thomas 1988; Pfennig 1992b; R. A. Martin, unpubl. data) This carnivore morphology is induced by ingestion of fairy shrimp (Pfennig 1990). However, the likelihood of becoming a carnivore also has a heritable basis. When raised under common conditions, families differ in their propensity to produce carnivore tadpoles (Pfennig & Frankino 1997; Pfennig 1999). This heritable component may be driven in part by maternal body size. Larger females tend to produce larger eggs and larger tadpoles. Larger tadpoles eat fairy shrimp faster, which suggests they are more likely to become carnivores (R. A. Martin & D. W. Pfennig, unpubl. data).

In the San Simon Valley of southeastern Arizona and southwestern New Mexico, *S. multiplicata* and *S. bombifrons* have undergone ecological character displacement in tadpole morph production. In ponds with only one *Spea* species present, both species produce similar, intermediate frequencies of each morph. However, in ponds where the two species co-occur, *S. multiplicata* produces nearly 100% omnivores, while *S.*

bombifrons produces nearly 100% carnivores (Pfennig & Murphy 2000, 2003; Pfennig *et al.* 2006). Experiments reveal that this divergence is driven by resource competition. Under common laboratory conditions, *S. multiplicata* omnivores were better competitors for detritus versus *S. bombifrons* omnivores (Pfennig & Murphy 2000). Likewise, *S. bombifrons* carnivores were better competitors for shrimp than *S. multiplicata* carnivores (Pfennig & Murphy 2000). Finally, *S. multiplicata* tadpoles raised with *S. bombifrons* tadpoles similar in resource use experienced more competition and performed more poorly than when raised with *S. bombifrons* less similar in resource use (Pfennig *et al.* 2007). Moreover, when raised under controlled conditions in the lab, *S. multiplicata* tadpoles with parents from ponds where S. bombifrons was less frequent were more likely to develop as carnivores versus tadpoles with parents from ponds where *S. bombifrons* is more frequent (Pfennig & Murphy 2002).

Ecological character displacement between *S. multiplicata* and *S. bombifrons* has a variety of consequences. First, this character displacement appears to have negative fitness consequences for *S. multiplicata* in sympatry. In part because they are displaced to the detritus resource in sympatry, which is less nutritious than fairy shrimp (Pfennig & Murphy 2000), adult *S. multiplicata* are significantly smaller in sympatry versus allopatry (Pfennig & Pfennig 2005). Smaller body size is associated with lower survival and fecundity (Pfennig & Pfennig 2005). Second, postmating isolation between *S. multiplicata* populations in sympatry versus allopatry has arisen as a by-product of competition with *S. bombifrons*. *S. multiplicata* in allopatry are under selection to produce both omnivore and carnivore phenotypes (Pfennig *et al.* 2007). In sympatry, however, *S. multiplicata* experience selection for a more omnivore-like phenotype

(Pfennig *et al.* 2007). Tadpoles produced by matings between individuals from sympatry and allopatry do not compete well in either parental environment, presumably because they have intermediate trophic phenotypes (Pfennig & Rice 2007).

Thanks to previous work in this system (e.g., Pfennig & Murphy 2000, 2002, 2003; Pfennig & Pfennig 2005; Pfennig *et al.* 2006, 2007), the process of ecological character displacement between *S. multiplicata* and *S. bombifrons* is well understood. With this dissertation research, I sought to investigate some of the factors that may affect this process, as well some possible consequences of ecological character displacement. To do so, I employed a population genetics approach, which has previously been used to only a limited degree in this system (Simovich 1985; Simovich & Sassaman 1986). This research will increase our understanding of how ecological character displacement proceeds, why and when certain outcomes may be more likely, and the role character displacement may play in speciation. Ultimately, by better understanding ecological character displacement, we will better understand the forces that generate biological diversity.

CHAPTER II

CHARACTER DISPLACEMENT: *IN SITU* EVOLUTION OF NOVEL PHENOTYPES OR SORTING OF PRE-EXISTING VARIATION?[1]

Summary

Character displacement – the divergence of traits between species in response to competition for resources or mates – has long been viewed as a major cause of adaptive diversification and species coexistence. Yet, we lack answers to basic questions concerning the causes and consequences of character displacement, not the least of which is why some species are more prone than others to undergo character displacement. Here, we address these questions by describing how character displacement can proceed through two nonexclusive routes that differ in the source of phenotypic variation, and, hence, in the ease with which character displacement may unfold. During *in situ* evolution of novel phenotypes, new traits that are divergent from a heterospecific competitor are generated and spread in sympatry. During sorting of pre-existing variation, such traits are initially favored in allopatry before the two species encounter one another. Later, when they come into contact, character displacement transpires when these pre-existing divergent phenotypes increase in frequency in sympatry relative to allopatry. Because such sorting of pre-existing variation should unfold relatively rapidly,

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[1] This chapter is modified from Rice, A. M. and Pfennig, D. W. 2007. Character displacement: *in situ* evolution of novel phenotypes or sorting of pre-existing variation? *Journal of Evolutionary Biology* 20: 448-459.

we suggest that species that express resource or mating polymorphism prior to interactions with heterospecifics may be more prone to undergo character displacement. We discuss the key differences between these two routes, review possible examples of each, and describe how the distinction between them provides unique insights into the evolutionary consequences of species interactions, the origins of diversity, and the factors that govern species coexistence.

Introduction

Character displacement (Brown & Wilson 1956), or what Darwin (1859 (1964)) called divergence of character, is a commonly observed pattern in plants and animals (reviewed in Howard 1993; Schluter 2000b; Dayan & Simberloff 2005). Populations of two closely related species are often different phenotypically where the species occur together ("sympatry") but are indistinguishable where each species occurs alone ("allopatry"; Fig. 2.1a).

Character displacement may take two distinct forms. First, when species compete for resources, selection may lead to "ecological character displacement" (Slatkin 1980; Schluter 2001). Ecological character displacement arises when competition between similar heterospecific individuals imposes directional selection on each species' resource use and associated phenotypic characters, leading to divergence between species in these traits and a concomitant reduction in competition (reviewed in Robinson & Wilson 1994; Schluter 2000b; Day & Young 2004; Dayan & Simberloff 2005). Second, when species interfere with each other's ability to identify conspecific mates, or when they risk costly mismatings with one another, selection may lead to "reproductive character

displacement" (Blair 1955; Crozier 1974). Reproductive character displacement arises when interactions between similar heterospecific individuals imposes directional selection on each species' mating signals or preferences, leading to divergence between species in these traits and a concomitant reduction in reproductive interference (reviewed in Howard 1993; Coyne & Orr 2004).

Although character displacement has long been viewed as a major factor in promoting species divergence, species coexistence, and adaptive radiation (Fig. 2.1; reviewed in Howard 1993; Schluter 2000b; Coyne & Orr 2004; Day & Young 2004), we lack answers to basic questions such as: What factors determine whether species interactions result in character displacement as opposed to competitive exclusion (Hardin 1960; Connell 1961)? Is character displacement invariably a slow process? What role does character displacement play in the origin of novel phenotypes? Why does character displacement sometimes ignite speciation and adaptive radiation and sometimes not? And, perhaps most fundamentally, why are some species more prone than others to undergo character displacement?

In this paper, we provide potential answers to these questions by describing how character displacement can proceed through two nonexclusive routes. These routes differ in the geographic source of phenotypic variation (i.e., allopatry or sympatry with a heterospecific competitor), and hence, in the ease with which character displacement may occur. Under one route, divergent traits that lessen resource competition or signal interference arise and then spread in sympatry following contact with the heterospecific competitor. Under the other route, selection in allopatry may lead to the evolution of phenotypes that are pre-adapted for, and therefore differentially spread in response to,

competition in sympatry. We suggest that this second route may make character displacement more likely to occur and may therefore be the more common route. We discuss the key differences between these two routes, review possible examples of each, and describe how the distinction between them provides unique insights into the evolutionary consequences of species interactions, the origins of diversity, and the factors that govern species coexistence.

We begin by describing a possible bias in the occurrence of character displacement. This bias suggests that character displacement may be more likely to occur when selection in allopatry leads to the evolution of divergent phenotypes that are predisposed to succeed in sympatry with heterospecific competitors.

A Possible Bias in Character Displacement

Although taxonomically widespread (Schluter 2000b), character displacement tends to be especially prevalent among species that are phenotypically variable (Milligan 1985), particularly those that express resource or mating polymorphism (Pfennig & Murphy 2002). For example, such polymorphism occurs in giant rhinoceros beetles, *Chalcosoma atlas* and *C. caucasus* (Kawano 2002), threespine stickleback fish, *Gasterosteus aculeatus* (Day *et al.* 1994), sunfish, *Lepomis gibbosus* and *L. macrochirus* (Robinson *et al.* 1993; Robinson & Wilson 1996), spadefoot toads, *Spea bombifrons* and *S. multiplicata* (Pomeroy 1981; Pfennig 1992a), red-backed salamanders, *Plethodon cinereus* (Maerz *et al.* 2006), and, potentially, numerous species of *Anolis* lizards (Losos *et al.* 2000) and northern postglacial fish (Robinson & Wilson 1994). When these species co-occur with closely related heterospecific competitors, they typically undergo character

displacement by shifting from producing two morphs to producing primarily the single morph that is less like the competing species [in *Chalcosoma atlas* and *C. caucasus* (Kawano 2002); *Gasterosteus aculeatus* (Schluter & McPhail 1992); *Lepomis gibbosus* and *L. macrochirus* (Werner & Hall 1976); *Spea bombifrons* and *S. multiplicata* (Pfennig & Murphy 2000); *Plethodon cinereus* (Adams & Rohlf 2000); *Anolis* (Losos *et al.* 2001); and northern postglacial fish (Robinson & Wilson 1994)].

We suggest that the greater prevalence of character displacement in species that express resource or mating polymorphism reflects a greater ease with which character displacement occurs in such species. Specifically, the presence of a resource or mating polymorphism may render species more prone to character displacement for three reasons.

First, character displacement may proceed more quickly in populations with resource or mating polymorphism because divergent phenotypes already exist in such systems. Models suggest that character displacement can be a slow process, particularly in populations that initially lack phenotypic variation (Slatkin 1980; Milligan 1985; Taper & Case 1985). Thus, adaptation to competitors is often limited by the rate at which new variants are created by mutation and/or recombination. If the rate at which new variants are created is low, and competition intense, competitive exclusion, rather than character displacement, will likely result (Milligan 1985; Pfennig *et al.* 2006). If, however, divergent phenotypes pre-exist in allopatry (e.g., as might be the case if the competing species already express resource or mating polymorphism), then character displacement may get a "jump-start" (Milligan 1985; Schluter 2000b, p. 128) and proceed more quickly once a heterospecific competitor is encountered.

Second, populations that express resource or mating polymorphism have already undergone a sort of "intraspecific character displacement" (*sensu* West-Eberhard 2003). Many of the mechanisms and conditions that produce and maintain resource or mating polymorphism are the same as those that underlie character displacement. In both cases, divergent phenotypes are produced in response to competitively-mediated selection (Robinson & Wilson 1994; Schluter 2000b; Day and Young 2004). Thus, populations that express resource or mating polymorphism are poised to respond rapidly when they encounter a heterospecific competitor because they have already been "tested" in competition.

Finally, alternative phenotypes that arise through phenotypic plasticity may be especially likely to undergo character displacement, because phenotypic plasticity facilitates character displacement. In many species, divergence between heterospecific competitors is mediated, at least in part, by competitively-mediated plasticity (e.g., see Werner & Hall 1976; Robinson & Wilson 1994; Pfennig & Murphy 2000; Losos *et al*. 2001). Although some contend that competitively-mediated plasticity is not "true" character displacement (Grant 1972; Endler 1986; Schluter & McPhail 1992; Schluter 2000b)—because one of the six widely-accepted criteria for character displacement is that phenotypic differences between populations and species should have a genetic basis (Grant 1972; Arthur 1982)—the magnitude and direction of a plastic response to the environment (the "norm of reaction") is often genetically variable (Schlichting & Pigliucci 1998) and subject to adaptive evolution (West-Eberhard 1989).

More importantly, phenotypic plasticity may promote character displacement by facilitating "valley crossing" (Pfennig *et al*. 2006). Consider a population that occupies

one of two possible peaks on an adaptive landscape (the two peaks might correspond to two morphs). If a superior competitor invades and begins to utilize the same limiting resource, the population would have to cross a fitness valley of maladaptive intermediate forms to climb the alternative peak (and use an alternative resource), a process normally prevented by natural selection. With phenotypic plasticity, however, populations can shift rapidly from one peak to the other without having to pass through the intervening selective valley (Kirkpatrick 1982; Schlichting & Pigliucci 1998; Pál & Miklos 1999). Such populations can express an alternative, selectively-favored phenotype that is unlike the competitor's without having to wait many generations for such adaptive phenotypes to arise through mutation or recombination (Pfennig & Murphy 2000, 2002). Without plasticity, a superior competitor may drive the focal species locally extinct before it has time to evolve new canalized traits that lessen competition.

Competitively-mediated plasticity might eventually lead to the evolution of "true" character displacement if divergent phenotypes become canalized under strong and persistent selection. Such canalization may occur, possibly through genetic assimilation (Waddington 1956) or genetic accommodation (West-Eberhard 2003), for two reasons. First, selection should become increasingly effective at producing a particular phenotype (as opposed to the alternative phenotype(s)) as that phenotype becomes increasingly common in the population (West-Eberhard 1989). Second, as one phenotype is expressed continuously in a population, and as the alternative phenotype is never expressed, alleles that regulate expression of this "hidden" phenotype would not be exposed to selection, and thus are at risk of chance loss (e.g., through drift or gradual mutation accumulation).

For example, tadpoles of spadefoot toads (*Spea multiplicata*) develop into two environmentally-triggered morphs: an omnivore morph that feeds on detritus at the pond bottom and a carnivore morph that feeds on anostracan fairy shrimp in open water (Pfennig & Murphy 2002). When these tadpoles encounter another species, *S. bombifrons*, that produces a competitively superior carnivore morph, they facultatively switch to producing mostly omnivores (Pfennig & Murphy 2002). Interestingly, *S. multiplicata* tadpoles from populations that historically have had more contact with *S. bombifrons* are canalized to produce all omnivores. Thus, competitively-mediated plasticity might often promote the rapid evolution of canalized character displacement.

In sum, character displacement tends to be especially prevalent among species that express resource or mating polymorphism, possibly because: (1) divergent phenotypes already exist in such systems; (2) these divergent phenotypes typically evolve in response to intraspecific competition and have therefore already been "tested" in competition; and (3) such alternative phenotypes often arise through phenotypic plasticity, and phenotypic plasticity may promote character displacement by facilitating "valley crossing." When such species encounter a closely related heterospecific competitor, they typically undergo character displacement by shifting (through phenotypic plasticity, canalization, or both) from producing two morphs to producing primarily the single morph that is less like the competing species.

Two Routes to Character Displacement

As the above discussion suggests, character displacement may evolve through two nonexclusive routes (Fig. 2.2). First, traits that differ from the competitor's and that

thereby lessen competition or reproductive interference may arise (through mutation, recombination, and/or hybridization) and then spread (through the action of competitively-mediated natural selection) in sympatry following contact with the competitor. This route, which we term "*in situ* evolution of novel phenotypes" (hereafter "ISE"), generates new phenotypes in sympatry that are not initially present in either species in allopatry. Second, divergent traits may be selectively favored in allopatry before interspecific competition takes place. As with ISE, such traits might spread through the action of competitively-mediated natural selection, albeit within species. Later, when the two species come into contact, character displacement occurs when these pre-existing divergent phenotypes increase in frequency in sympatry relative to allopatry. This second route, which we term "sorting of pre-existing variation" (hereafter "sorting"), selectively filters divergent phenotypes in sympatry that were already present in allopatry (as might be the case in populations that express resource or mating polymorphism).

As we describe below (see "Case Studies"), ISE and sorting are not mutually-exclusive and may occur simultaneously or sequentially (Schluter & Grant 1984; Schluter 2000b; Marko 2005). Sorting may operate first, with ISE following and magnifying the pre-existing differences between species (Schluter 2000b, p. 128).

Most researchers do not consider sorting an alternative route to the evolution of character displacement (e.g., Slatkin 1980; Arthur 1982; Taper & Case 1985; Doebeli 1996; but see Endler 1986, p. 62; Thompson 1994, p. 248; Pfennig & Murphy 2003; Marko 2005). Although sorting between species has been widely discussed as a mechanism for community-wide character displacement (reviewed in Dayan &

Simberloff 2005), the possibility that sorting might function within species to promote character displacement is seldom considered.

Because selection must initially act on standing variation, it might be contended that all cases of character displacement begin as sorting (e.g., in Fig. 2.2b, individuals of the focal species that are in the left tail of the distribution will be selectively favored through a process similar to sorting). However, ISE goes beyond this initial sorting process and favors novel and increasingly divergent phenotypes in sympatry (Fig. 2.2c). When resource or mating polymorphism is present in allopatry, character displacement due to the sorting of pre-existing variation *alone* may be sufficient to avoid interspecific competition (Fig. 2.2f).

In the next section, we explain how the distinction between ISE and sorting has important implications for the evolution of novel phenotypes and the likelihood that character displacement may promote ecological speciation and adaptive radiations. Indeed, as we will show, the distinction between these two routes is critical for predicting whether character displacement will occur in the first place.

Evolutionary Implications of the Two Routes to Character Displacement

Although both sorting and ISE promote character divergence in the face of competition, the two processes differ in how and under what circumstances they promote character displacement (Table 2.1). These differences have important evolutionary implications for understanding: (1) why character displacement occurs in some situations but not in others; (2) the speed with which character displacement evolves; (3) the

ultimate factors that generate divergent phenotypes; and (4) the likelihood that character displacement will ignite ecological speciation or adaptive radiation.

Distinguishing between ISE and sorting may help explain why character displacement occurs in some situations but not others. Contrary to sorting, with ISE, new phenotypes that differ from those of ancestral pre-displacement populations (allopatry) are selectively favored in sympatry. Exploitable resources (or, in the case of reproductive character displacement, signal space) beyond those in allopatry must therefore be available for this process to occur; i.e., a superior competitor should not already utilize these resources. In the absence of such exploitable resources, competitive exclusion, rather than ISE mediated character displacement, may result (Pfennig *et al.* 2006). If a population already utilizes an alternative resource, even at low frequencies, it may be better poised to take advantage of that resource when faced with competition for its primary resource (Fig. 2.2d-f). Sorting may therefore be a more likely mechanism for character displacement in "saturated" communities; i.e., species-rich communities that contain relatively few underexploited niches. In such communities, novel phenotypes arising through ISE may be unsuccessful because of a dearth of available, underutilized resources. By contrast, novel phenotypes arising through ISE may be more successful when there are a wide variety of resources to exploit with few competitors, as may be the case, for example, following mass extinctions or the colonization of new habitats. In such settings, few competitors would be present, and underutilized resources would therefore be available to permit the evolution of new resource-use phenotypes that are required for ISE to unfold (Fig. 2.2c).

Differentiating ISE from sorting may also help explain the speed of character displacement (Fenchel 1975; Diamond *et al.* 1989; Pfennig & Murphy 2002, 2003). Traditionally, character displacement was thought to be a slow process (Slatkin 1980; Taper & Case 1985), limited by the rate at which divergent traits arise and spread in sympatry. If species are initially similar phenotypically, and the rate at which divergent traits are introduced is low but competition intense, competitive exclusion may result (Milligan 1985). If, however, divergent traits pre-exist in allopatry (as with sorting; Fig. 2.2), then character displacement will likely proceed more quickly than if it were driven entirely by ISE. Sorting may therefore "buy" time and enable competing species to co-exist long enough for ISE to produce new variation in sympatry that amplifies differences between competitors. Thus, because sorting should transpire more rapidly, this route may be primarily responsible for character displacement in systems with recent sympatric contact. By contrast, ISE may operate primarily in systems with more ancient sympatry.

Sorting and ISE also differ in the ultimate agents of selection that generate competitively-mediated phenotypes. During ISE, the agent of selection that favors divergent phenotypes is interspecific competition. By contrast, during sorting, divergent phenotypes evolve in allopatry, prior to contact with the competitor. In this case, the agents of selection that favor divergent phenotypes are forces that act in allopatry. Intraspecific competition, for example, might initially select for alternative resource use or mating tactic morphs (Pfennig 1992a; Hori 1993; Maret & Collins 1997; reviewed in Gross 1996; Smith & Skúkason 1996; West-Eberhard 2003). Later, when two such polymorphic species come into contact, character displacement occurs when these pre-existing divergent phenotypes increase in frequency in sympatry relative to allopatry.

Thus, in contrast with ISE, for sorting, divergent phenotypes that lessen competition between species are not initially favored because of *interspecific* competition.

Finally, whether character displacement arises through ISE or sorting may dictate whether sympatric and allopatric populations within a species diverge to the point of triggering ecological speciation (Fig. 2.1b; for a general review of ecological speciation, see Rundle & Nosil 2005). Character displacement can ignite ecological speciation if sympatric and allopatric populations diverge to such a degree that any offspring produced by matings between such populations have lower fitness than those produced within populations. If character displacement arises via ISE, novel phenotypes in sympatry are much more likely to be incompatible with those in allopatry. Such incompatibility between sympatric and allopatric populations may favor the evolution of isolating mechanisms between these populations. Sorting, by contrast, results in sympatric phenotypes that are a subset of those already present in allopatry. Therefore, if character displacement arises through sorting, phenotypes in sympatry are much less likely to be incompatible with those in allopatry. As a result, sorting should be less likely than ISE to promote the evolution of reproductive isolation and speciation. Indeed, adaptive radiations, by definition, are unlikely to arise by sorting, because novel phenotypes are not generated.

Case Studies

Below, we outline a series of case studies that potentially illustrate how character displacement can arise through ISE or sorting. For each example, we inferred the signature of each route by comparing the population mean phenotypes in sympatry with

the range of phenotypes present in allopatry. We reasoned that if character displacement evolved through sorting, then phenotypes in sympatry would be within the range of those in allopatry (Pfennig & Murphy 2003). Alternatively, we reasoned that ISE would account for character displacement if sympatric population phenotypic means were more extreme than allopatric population phenotypic ranges for a given example (e.g., see Fig. 2.2). When phenotypic range data were not available, we compared sympatric phenotypic means to allopatric standard deviations (e.g., *Hydrobia* snails, Fenchel 1975) or allopatric standard errors (e.g., *Spea* toads, Pfennig & Murphy 2003). Such a comparison is more likely to implicate ISE and less likely to implicate sorting than a comparison of sympatric means to allopatric ranges, because the allopatric phenotypic range would be broader than the allopatric mean ± one standard deviation or standard error.

Although we used a comparison of sympatric means to allopatric ranges to infer the signatures of sorting and ISE in the following examples, when raw data are available, a comparison of trait variances between populations in sympatry and allopatry may also be employed. When sorting involves a shift from producing two morphs in allopatry to producing primarily the single morph that is less like the competing species, trait variance in sympatry should be reduced relative to the variance in allopatry (compare Fig. 2.2d with Fig. 2.2f). In contrast, with ISE, because new phenotypes are selectively favored in sympatry, trait variance in sympatry may not be reduced relative to allopatry. The variance ratio test (Zar 1999) can be used to determine whether the variance in sympatry is reduced relative to allopatry or not. This test may be preferable to a comparison of sympatric means to allopatric ranges because it may be used for multivariate data.

However, like the comparison of means to ranges, the variance ratio test cannot conclusively distinguish between ISE and sorting for two reasons: first, although likely to be less drastic, ISE may also show a reduction in variance in sympatry due to the action of selection; and second, in some sorting situations (e.g., when both morphs from allopatry are present in sympatry, but have reversed frequencies, such as if morph 1 from Fig. 2.2d increased to the original morph 2 frequency after selection, and vice versa for morph 2), variance between allopatry and sympatry may not be reduced (e.g., see Pfennig & Murphy 2003). Because these scenarios are not likely to be common, the variance ratio test is still useful as an initial analysis. Along with a comparison of sympatric means to allopatric ranges, this preliminary test may then be followed with more rigorous testing (see "Suggestions for Future Research"). Because the raw data in three of the following four examples were unavailable to us, we were only able to perform the variance ratio test on the *Spea* toads.

The following examples highlight two key predictions outlined in the previous section. First, because sorting should precede ISE in the evolution of character displacement, sorting should be more common in species that have come into contact and undergone character displacement relatively recently. Second, species that express resource or mating polymorphism prior to interactions with heterospecifics should be more likely to undergo character displacement through sorting.

Galapagos Finches

Two species of ground finch on the Galapagos Islands, *Geospiza fortis* and *G. fuliginosa,* exhibit divergence in beak depth on sympatric islands, but possess similar

beak depths on allopatric islands (Lack 1947). Beak depth has been linked to preferred seed size, and competition for resources (seeds) appears to be responsible for divergence of the beak depth phenotype in sympatry (Lack 1947; Schluter & Grant 1984). The first sympatric contact between these two species likely occurred in the last 80,000 years, sometime after the split between *G. fortis* and *G. fuliginosa* (Yang & Patton 1981; Grant 1994).

Sorting may be primarily responsible for the character displacement in *G. fuliginosa*, while ISE has likely been acting in *G. fortis*. In *G. fuliginosa,* data from Lack (1947) indicate that mean beak depths for 8 of 10 sympatric islands lie within the range of beak depths present on Los Hermanos, the allopatric island habitat of *G. fuliginosa*. However, in *G. fortis*, all 10 population beak depth means in sympatry lie outside the range of beak depths in allopatry (Daphne), suggesting that ISE has been operating in this species.

This example therefore illustrates how ISE and sorting potentially operate to generate character displacement. More critically, this example underscores that ISE and sorting may operate independently within each interaction; i.e., one species in a competitive interaction can undergo character displacement through sorting, whereas the other can undergo character displacement through ISE.

Hydrobia Snails

Shell lengths for two mud snail species in the Limfjorden, Denmark, *Hydrobia ulvae* and *H. ventrosa,* have diverged in sympatric populations, but not in allopatric populations (Fenchel 1975). Food particle size corresponds to shell length (Fenchel 1975; Fenchel & Kofoed 1976), and these species exhibit interspecific competition and partition resources

based on size (Fenchel & Kofoed 1976; Gorbushin 1996). In addition, this sympatric divergence in shell length has occurred within no more than 175 generations, since the presence of these species in this fjord, and hence their contact, postdates 1825 (Fenchel 1975).

Sorting appears to be primarily responsible for the evolution of character displacement in these two species, although there is evidence of ISE in some populations of *H. ulvae*. In *H. ventrosa*, shell length means for all sympatric locations fall within one standard deviation of the mean for 7 of 8 allopatric locations. This pattern is not quite as strong for *H. ulvae*, in which 8 of 15 sympatric population means lie within one standard deviation of the allopatric means, suggesting sorting, while 7 of 15 sympatric populations average shell lengths greater than one standard deviation above the allopatric means, suggesting ISE.

As in the previous example, each species differs in whether ISE or sorting accounts for character displacement. Moreover, both sorting and ISE can contribute to trait evolution in the same population.

Giant Rhinoceros Beetles

Body size and genitalia length in two Southeast Asian giant rhinoceros beetle species, *Chalcosoma caucasus* and *C. atlas*, exhibit divergence in sympatry relative to allopatry (Kawano 2002). These species show male dimorphism, with a large-bodied, long-horned major morph, and a smaller-bodied, short-horned minor morph, which likely reflects alternative behaviors for finding mates (Kawano 2002 and references therein). Moreover, body size is highly variable within populations, while genitalia length is not

(Kawano 2004). Whether morphs are analyzed separately or together, divergence between sympatry and allopatry in body size and genitalia length remains significant (Kawano 2002). Sympatric differentiation in overall body size may reflect selection to avoid interspecific combat, whereas divergence in genitalia length likely reflects selection to avoid hybridization (Kawano 2002). It is unknown how long these species have been sympatric.

Sorting may mediate divergence in body size, whereas ISE may mediate divergence in genitalia size. For *C. caucasus*, mean body size for all sympatric populations falls within the ranges of the 3 allopatric populations. Likewise, for *C. atlas*, all 7 sympatric means fall within the ranges of 8 out of 9 allopatric populations, suggesting sorting. In contrast, for genitalia length, all sympatric population means for *C. caucasus* lie outside 2 of the 3 allopatric ranges, while all *C. atlas* sympatric population means fall outside the ranges of 4 of 9 allopatric locations (data from Kawano 2002). This pattern suggests that ISE has acted on genitalia length.

This example indicates that ISE and sorting may operate independently on different traits within a single population. When one trait exhibits more variation within the population than another trait, such as body size in this example, sorting on the more variable trait may "jump-start" character displacement, quickly reducing competition between species. This initial reduction in competition may allow coexistence long enough for variation to arise in another trait, which may subsequently diverge through ISE. Thus, not only can both ISE and sorting operate independently between species, as in the Galapagos finches and *Hydrobia* snails examples, but they can operate independently on different traits within species as well.

Moreover, this example confirms our prediction that species that express polymorphism prior to interactions with heterospecifics should undergo sorting. These beetles are dimorphic in body size in allopatry. Although both major and minor morphs are present in sympatry (likely reflecting intraspecific competition for mates), the combined body size range for both morphs of one species in sympatry approximately corresponds with the body size range for one morph in allopatry. This pattern suggests the divergence in body size has evolved by sorting, as predicted.

Spea Toads

As noted above ("A Possible Bias in Character Displacement"), two species of spadefoot toad, *Spea multiplicata* and *S. bombifrons*, diverge in tadpole morph production in mixed-species ponds (syntopy) relative to pure-species ponds (allotopy) in the southwestern United States (Pfennig & Murphy 2000, 2003). In southeastern Arizona (where much of the work on these two species has been conducted), sympatry has likely occurred within the last 150 years (Pfennig *et al.* unpubl. data).

Morphological (Pfennig & Murphy 2003; Pfennig *et al.* 2006) and comparative population genetic (Rice and Pfennig unpubl. data) data failed to provide evidence of sorting in this system. Using four trophic characters as an indication of morph production, Pfennig and Murphy (2003) found that all syntopic population means for three of the characters in *S. multiplicata* lie outside the standard errors of the mean in at least 10 of 13 allotopic populations, which is consistent with ISE. Likewise, all syntopic population means (except one trait mean in one population) for three characters in *S. bombifrons* were outside the standard errors for all of the allotopic populations (Pfennig

& Murphy 2003), again pointing to ISE. Moreover, for both species, no differences were found between syntopic and allotopic variances in pond means for a composite shape variable reflecting three trophic characters (*S. multiplicata*: $F_{16,6} = 1.34$, $P = 0.76$; *S. bombifrons*: $F_{7,4} = 1.48$, $P = 0.74$; data re-analyzed from Pfennig *et al.* 2006), providing further support for ISE. Reinforcing these morphological results, a comparative population genetic analysis of *S. multiplicata* employing a partial Mantel test indicated that the divergence in morph production between syntopic and allotopic populations cannot be accounted for by genetic distance between these populations (Rice and Pfennig unpubl. data), as would be expected if sorting were important (see below).

Thus, at first glance, the lack of evidence for sorting would seem to run counter to our prediction that species (such as *Spea*) that express resource polymorphism prior to interactions with heterospecifics should undergo character displacement through sorting. Experiments reveal, however, that divergence between competitors in this system is mediated (at least in part) by phenotypic plasticity (Pfennig & Murphy 2000, 2002). Such competitively-mediated phenotypic plasticity can be even faster than sorting in promoting phenotypic differences between species, thereby lessening the need for character displacement to evolve through sorting (Pfennig *et al.* 2006).

Summary of Case Studies

The above case studies suggest that character displacement can evolve through either ISE or sorting. Indeed, different routes may promote character displacement among different species in the same competitive interaction (as in *Geospiza* finches) or even among different traits in the same species (as in *Chalcosoma* beetles).

These case studies also suggest that ISE and sorting differ in the speed with which they promote character displacement. Because sorting should precede ISE in the evolution of character displacement, we predicted that sorting may be responsible for the relatively rapid evolution of character displacement in systems with recent sympatric contact, while ISE may be important for magnifying interspecific divergence in systems with more ancient sympatry. As predicted, in species that have come into contact recently (e.g., *Hydrobia* snails), character displacement appears to have evolved rapidly through sorting. By contrast, in species that have been in contact relatively long (e.g., *Geospiza* finches), ISE appears to have played a major role in at least one of the species. Thus, as predicted, sorting may be important in "jump-starting" character displacement. Once enough time has passed for new variation to arise in sympatry, ISE may become more important as it further lessens competition or reproductive interference and magnifies the differences between species in sympatry.

Spea toads appear to run counter to the prediction that sorting promotes character displacement in systems with recent sympatric contact. Although competitors likely came into contact relatively recently, sorting does not appear to be important in driving character displacement. As noted above, there is no need for sorting, because phenotypic plasticity mediates the early divergence between sympatric competitors (Pfennig & Murphy 2002). As with sorting, phenotypic plasticity may "jump-start" the process of character displacement, preventing competitive exclusion before new variation has time to arise (Pfennig & Murphy 2002; Pfennig *et al.* 2006).

Our second prediction was that species that express resource or mating polymorphism prior to interactions with heterospecifics should be more likely to undergo character

displacement through sorting. *Chalcosoma* beetles satisfy this prediction. In addition, because sorting of pre-existing variation should unfold relatively rapidly, we predicted that polymorphic species should be predisposed to undergo character displacement in the first place. Although a cursory review of the literature suggests that character displacement does indeed seem to occur more frequently among species that express alternative morphs (see "A Possible Bias in Character Displacement"), additional studies are needed to evaluate this prediction more generally.

Finally, this overview demonstrates that alternative morphs are not necessary for sorting to occur, nor does the presence of alternative morphs ensure that character displacement will evolve via sorting. For instance, character displacement has likely evolved primarily through sorting in the finch *G. fuliginosa* and in both species of mud snail, *H. ulvae* and *H. ventrosa*. Yet, none of these species exhibits alternative phenotypes, suggesting that sorting may also occur in populations expressing continuously distributed phenotypes. Moreover, the presence of alternative morphs does not ensure that character displacement will evolve via sorting if, as in *Spea*, phenotypic plasticity mediates divergence.

Thus: (1) character displacement can evolve through ISE, sorting, phenotypic plasticity, or some combination, (2) both sorting and phenotypic plasticity may "jump-start" character displacement, (3) character displacement may proceed extremely rapidly if initiated by phenotypic plasticity, and 4) sorting is a general mechanism that applies to discrete or continuously distributed phenotypes.

Suggestions for Future Research

In the examples above, data on phenotypic means and ranges in sympatry versus allopatry enabled us to determine if sympatric means lie within (consistent with sorting) or outside (consistent with ISE) allopatric ranges. Such data are typically available from studies of character displacement and so can generally be used to ascertain for a given system how character displacement arises. Additionally, if raw data are available, a comparison of trait variance between sympatric and allopatric populations can provide an additional test to distinguish between sorting and ISE (see "Case Studies"). Because ISE and sorting are not mutually-exclusive, however, such analyses cannot establish which route is primarily responsible for the case of character displacement.

In combination with phenotypic data, genetic marker data can provide a powerful tool for evaluating which route leads to character displacement. Intraspecific independent contrasts (Felsenstein 2002), partial Mantel tests (Thorpe *et al.* 1995; Thorpe 1996), and spatial autocorrelation (Edwards & Kot 1995; Marko 2005) utilize estimates of gene flow (intraspecific independent contrasts) or genetic distance (partial Mantel tests and spatial autocorrelation) to determine if population history can account for the observed phenotypic divergence between sympatry and allopatry (expected for sorting), or if most or all of the divergent phenotypes arose and spread after contact was established in sympatry (expected for ISE). These analyses are comparative, however, and therefore cannot establish a causal link between the presence of the competitor and phenotypic divergence. Moreover, the signatures of sorting and founder effects will be similar in these analyses. Evidence of interspecific competition (e.g., Fenchel & Kofoed 1976; Gorbushin 1996; Pfennig & Murphy 2000, 2002) and/or selection for character displacement in sympatry (e.g., Pacala & Roughgarden 1985; Schluter 1994; Pritchard &

Schluter 2001; Gray & Robinson 2002) is therefore necessary to establish that competition promotes divergence and that differences between sympatry and allopatry are not attributable to chance founder events.

If it is possible to link genetic or phenotypic markers to specific groups of populations, populations, families, or even individuals *and* also to a particular resource use spectrum (*sensu* Day & Young 2004) or signal use spectrum, one could test whether certain markers, and therefore certain resource use or signal use phenotypes, are overrepresented in sympatry compared to allopatry, an expected signature of sorting. If so, experiments in controlled conditions could be performed to determine if the overrepresented groups tend to have a resource or signal use spectrum less like the competing species than expected by chance. Such an outcome would support a major role for sorting in character displacement.

Additionally, if genetic markers and genes affecting phenotypes associated with resource use are physically linked, Tajima's D (Tajima 1989) could be calculated for sympatry versus allopatry in order to determine the relative importance of ISE versus sorting. This analysis would gauge the relative strength of the signature of selective sweeps— very low levels of neutral variation linked to the trait under selection— in each region. If ISE has been more important, there should be no signature of a selective sweep in allopatry, while there should be a strong signature of a sweep in sympatry. Alternatively, if sorting has been important, there should be evidence of sweeps in both sympatry and allopatry. The signature in allopatry may be weaker, however, because selection for the divergent phenotype in allopatry should predate the selection in sympatry, allowing more time for the recovery of linked neutral variation. Moreover, if

the sympatric contact is ancient, any evidence of a selective sweep in allopatry may have been erased by the subsequent build-up of linked neutral variation over time ("old" sorting). Such a genetic analysis should therefore be accompanied by either an analysis of whether or not the sympatric phenotypic means extend beyond the range of allopatric phenotypes or a variance ratio test comparing allopatric and sympatric phenotypic variance. Doing so should effectively differentiate between the two routes to character displacement.

Because sorting and ISE are not mutually exclusive, both may play a critical role in generating patterns of character displacement. Yet, the above analyses may help determine which route has been predominant in any given case of character displacement. Moreover, meta-analyses can be employed to determine whether ISE or sorting generates the general patterns that we have described above. For example, such analyses can be used to determine whether polymorphic species are more likely than monomorphic species to undergo character displacement and coexist with competitors. This information may ultimately help clarify why some species are more prone than others to undergoing character displacement.

Finally, although we have focused on ecological character displacement (trait evolution resulting from selection to minimize resource competition between species), the same principles apply to reproductive character displacement (trait evolution resulting from selection to minimize reproductive interference between species). Future studies should test these predictions for reproductive character displacement.

Conclusion

Character displacement proceeds through two nonexclusive routes, which differ in the geographic source of phenotypic variation (i.e., allopatry or sympatry with the competitor), and hence, in the ease with which character displacement may occur. During in situ evolution of novel phenotypes, newly divergent traits arise and are favored in sympatry. During sorting of pre-existing variation, such traits initially arise and are favored in allopatry. Later, when competitors come into contact, character displacement transpires rapidly when these pre-existing divergent phenotypes increase in frequency in sympatry relative to allopatry. Modern molecular tools and phylogenetic or population genetic approaches may help differentiate between these two routes in different examples of character displacement. Such studies promise to provide unique insights into the evolutionary consequences of species interactions, the origins of diversity, and the factors that govern species coexistence.

Table 2.1. Summary of differences between the two routes to character displacement.

Route	**Geographic source of divergent phenotypes**	**Ultimate selective agent(s) promoting divergent phenotypes**	**Relative speed with which character displacement evolves**
in situ evolution	sympatry	interspecific resource competition or reproductive interference	slow
sorting	allopatry	various, but often intraspecific competition for resources or mates	fast

Table 2.1. (continued).

Route	**Situations in which route is is likely to be most common**	**Likelihood of triggering ecological speciation or adaptive radiation**
in situ evolution	unsaturated communities that have numerous open niches	high
sorting	either unsaturated or saturated communities	low

Figure Legends

Figure 2.1. Character displacement promotes diversity and coexistence between close competitors, and may even promote the origin of new species. (a) When two species compete and overlap in only part of their geographical range, they are often recognizably different where they occur together and indistinguishable where each occurs alone. The evolution of such exaggerated phenotypic differences in sympatry may reflect selection to minimize competition for shared resources (ecological character displacement) or to lessen the risk of hybridization or reproductive interference (reproductive character displacement). (b) Regardless of the precise cause of such divergence, because conspecific populations in sympatry and in allopatry with the competitor experience different selective regimes, character displacement may promote the origin of new species and, possibly, an adaptive radiation (indicated here by the formation of new species 3 and 4).

Figure 2.2. Two routes to character displacement: (a-c) *in situ* evolution of novel phenotypes, and (d-f) sorting of pre-existing variation. Initially (a, d), a focal species (species 1) occurs alone in allopatry, either as a monomorphic species (a) or as a polymorphic species (d) consisting of alternative resource use or mating tactic morphs (morphs 1, 2), one of which is initially rarer than the other(s). Later (b, e), a superior competitor, species 2 (heavy line), comes into sympatry with species 1 (either because species 2 invades the habitat of species 1 or vice versa). Finally (c, f), because of selection imposed by species 2, species 1 undergoes an evolutionary shift in resource use and associated phenotypic features (ecological character displacement) or in mating

signals/preferences (reproductive character displacement; in both cases, the distributions of species 1 before selection are shown in dashed lines). With *in situ* evolution of novel phenotypes (c), character displacement unfolds when novel phenotypes that are more dissimilar to the competitor spread in sympatry following the invasion of species 2. Because they are associated with reduced competition, these new phenotypes are selectively favored. As a result, the entire distribution of species 1 shifts to the left; i.e., away from the competitor. By contrast, with sorting of pre-existing variation (f), character displacement unfolds when the morph that is more dissimilar to the competitor (here, morph 1) is selectively favored and thereby increases in frequency at the expense of the alternative morph. As a result, the entire distribution of species 1 again shifts to the left. Although we have illustrated sorting of pre-existing phenotypes as involving discrete morphs, it could also occur in populations expressing continuously distributed phenotypes. In both cases (c, f), the outcome of character displacement is identical, even though the two populations undertook two different routes.

Figure 2.1

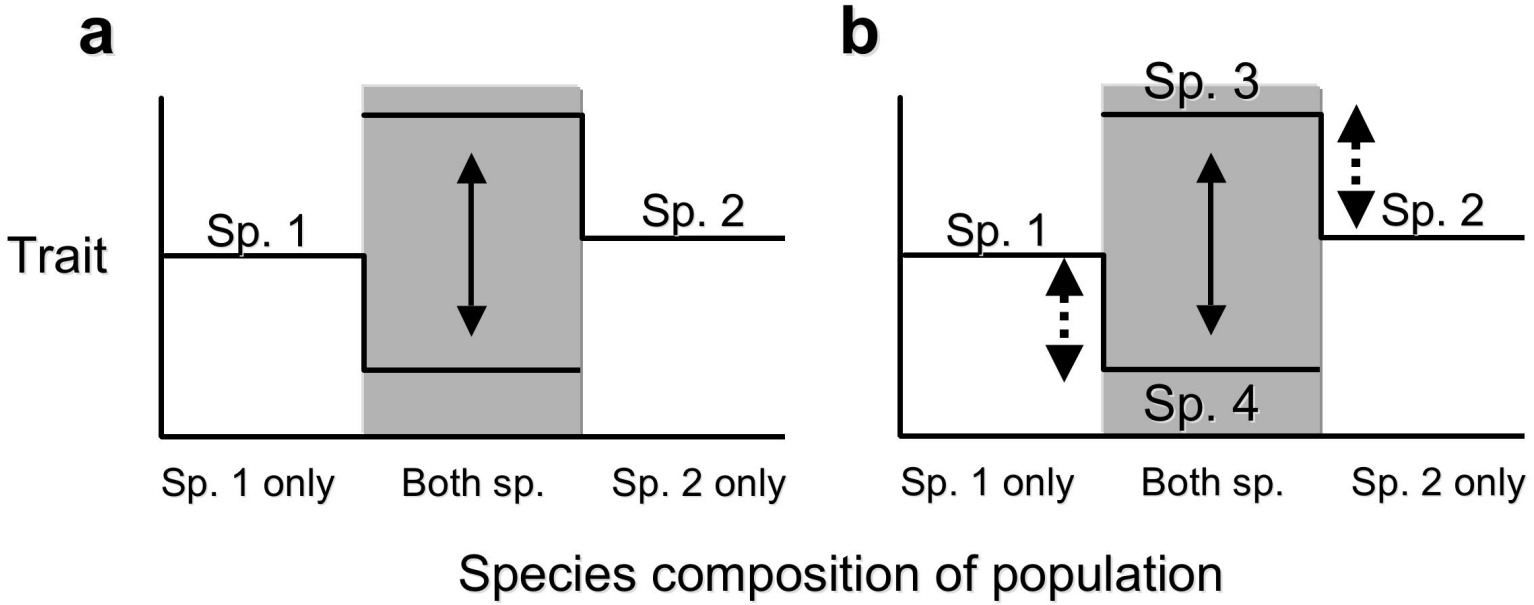
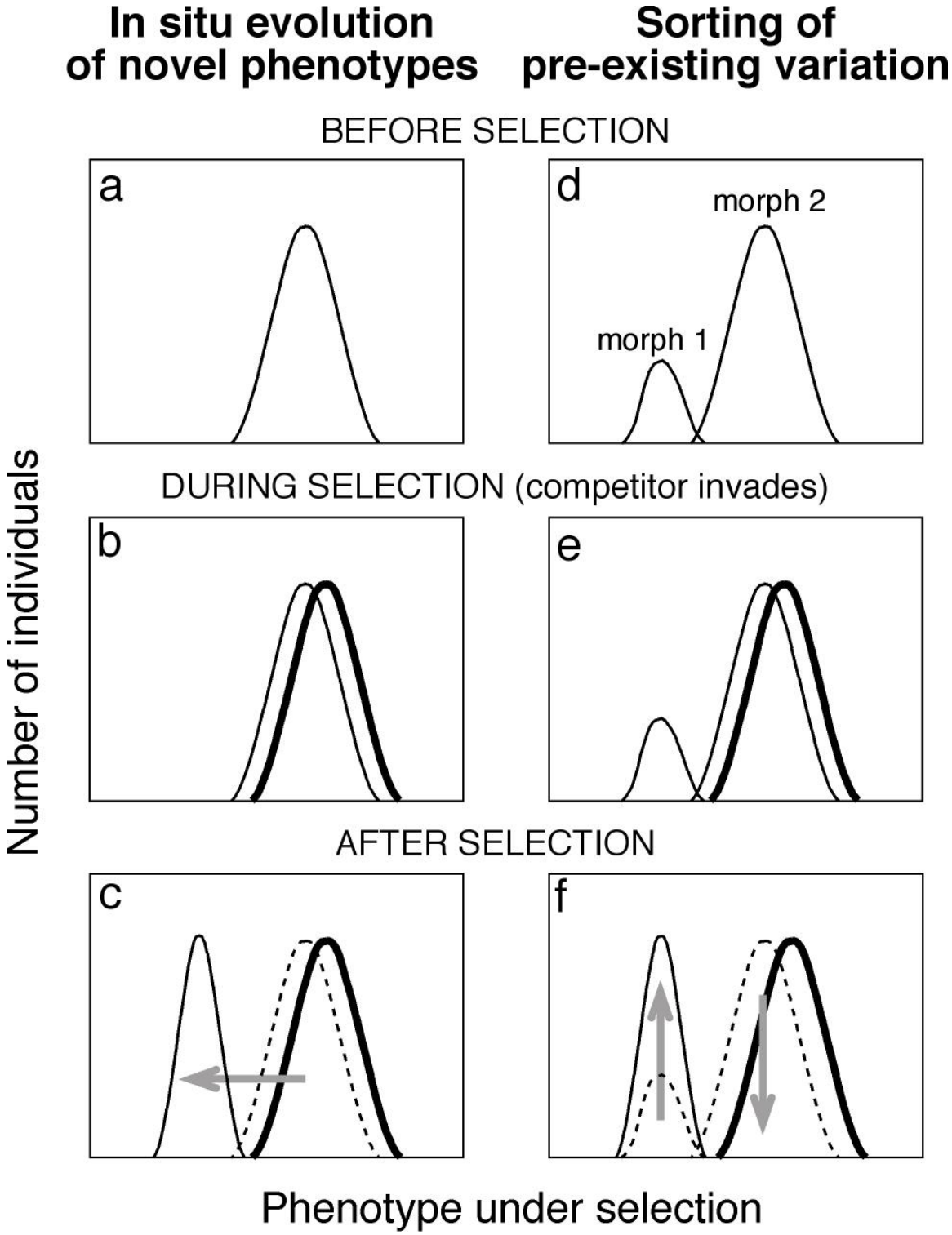

a
b
Trait
Sp. 1
Sp. 2
Sp. 3
Sp. 4
Sp. 1 only
Both sp.
Sp. 2 only
Species composition of population

Figure 2.2


In situ evolution of novel phenotypes
Sorting of pre-existing variation
BEFORE SELECTION
a
d
morph 2
morph 1
DURING SELECTION (competitor invades)
b
e
AFTER SELECTION
c
f
Number of individuals
Phenotype under selection

CHAPTER III

ANALYSIS OF RANGE EXPANSION IN TWO SPECIES UNDERGOING CHARACTER DISPLACEMENT: WHY MIGHT INVADERS GENERALLY 'WIN' DURING CHARACTER DISPLACEMENT?[2]

Summary

Ecological character displacement occurs when interacting species diverge in resource use and associated traits in response to selection to minimize resource competition between them. Yet, when resource quality is asymmetric, the species that monopolizes the more profitable resource following character displacement may have higher fitness and therefore be deemed the "winner". Here we ask: does the winner tend to be the resident species (i.e., the earlier inhabitant of the geographic region where character displacement occurred) or the invader (i.e., the subsequent inhabitant of the region)? We focus on two spadefoot toad species that have undergone character displacement. Previous studies revealed that *Spea bombifrons* gains the higher quality resource following character displacement; consequently, *S. multiplicata* must use the lower quality resource, and as a result, experiences negative fitness consequences. Where the two species have undergone character displacement, three lines of evidence implicate *S. bombifrons* as the invader: *S. bombifrons* possess lower haplotype and nucleotide

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[2] This chapter is modified from Rice, A. M. and Pfennig, D. W. 2008. Analysis of range expansion in two species undergoing character displacement: Why might invaders generally 'win' during character displacement? *Journal of Evolutionary Biology* 21: 696-704.

diversity; they do not exhibit isolation by distance (in contrast to *S. multiplicata*); and they display much higher population growth rates. We hypothesize that historical patterns of selection in its ancestral range pre-adapted *S. bombifrons* to evolve phenotypes capable of monopolizing the superior resource. Generally, because superior competitive abilities may facilitate successful invasions, invaders may be well positioned to win during character displacement.

Introduction

Ecological character displacement occurs when competition between interacting species imposes divergent directional selection on each species' resource use and associated traits, causing them to diverge in these characters (Grant 1972; Adams & Rohlf 2000; Schluter 2000b; Day & Young 2004; Rice & Pfennig 2007). One consequence of character displacement is that interacting species will evolve to utilize different resources, which can sometimes create a "winner" and a "loser." In particular, when asymmetries exist in resource quality, the species that monopolizes the higher quality resource will potentially have higher fitness (and may therefore be deemed the winner) compared to the species that is displaced from this resource (the loser; Pfennig & Pfennig 2005). These two species may enter into competition with each other through multiple scenarios: 1) sympatric speciation; 2) reciprocal expansions into a new geographical region; or 3) one species expanding into a geographic region already inhabited by the competitor. In cases of character displacement ignited by the last scenario, we ask: does the winner of character displacement tend to be the resident

species (i.e., the earlier inhabitant of the geographic region where character displacement occurred) or the invader (i.e., the subsequent inhabitant of the region)?

There are theoretical reasons for predicting that either the resident or the invader may win during character displacement. Residents might generally win if they tend to have longer association with a more profitable resource, and, consequently, if they were pre-adapted to monopolize this resource in the face of competition. By contrast, if the success of an invasion depends on the invading species' superior competitive ability (Sakai *et al.* 2001; Vila & Weiner 2004; but see Bossdorf *et al.* 2004), invaders might generally win in character displacement. For example, compared to noninvasive resident species, invasive species may forage more efficiently (Petren & Case 1996; Holway 1999; Rehage *et al.* 2005), convert resources into tissue growth more effectively (Byers 2000), or actively displace competitors from shared resources (Holway 1999). Because so little is known about whether residents or invaders are more likely to win during character displacement, a critical first step in understanding why one species is able to monopolize the more profitable resource in the face of competition is to establish whether the winner in character displacement is the invader or the resident.

We used spadefoot toads as a model system to investigate whether the invader or resident species wins during character displacement. As we describe below, tadpoles of two species (*Spea multiplicata* and *S. bombifrons*) have undergone ecological character displacement in southeastern Arizona (SE AZ) and southwestern New Mexico (SW NM), USA (Pfennig & Murphy 2000; Pfennig & Murphy 2003; Pfennig *et al.* 2006). Additionally, because the resources the two species use following character displacement are asymmetric in quality, the species that uses the lower quality resource, *S.*

multiplicata, is apparently experiencing negative fitness consequences of character displacement (Pfennig & Pfennig 2005). Although displacement to the lower quality resource is better for *S. multiplicata* than competitive exclusion, the fitness costs of this displacement may increase the risk of eventual Darwinian extinction of this species in sympatry (Pfennig & Pfennig 2005). Thus, *S. bombifrons* may be deemed the winner of this competitive interaction.

We used population genetic, phylogenetic, and phylogeographic analyses to address two issues. First, we asked which species was the invader into SE AZ and SW NM: *S. bombifrons* (the winner) or *S. multiplicata* (the loser)? Second, after determining that *S. bombifrons* was the invader into this region, we sought to determine its ancestral range. Estimating the ancestral range provided insight into historical patterns of selection that may have predisposed this species to monopolize the superior resource following character displacement.

Study system

Mexican spadefoot toads, *S. multiplicata*, and Plains spadefoot toads, *S. bombifrons*, co-occur in the southwestern US (Fig. 3.1). In a broad region of potential sympatry, both species may co-occur at intermediate elevations (hereafter termed "syntopy"). However, at high elevations, only *S. multiplicata* is present (hereafter termed "allotopy"), and at low elevations, only *S. bombifrons* is present (Pfennig *et al.* 2006). Phylogenetic hypotheses suggest that among the four currently recognized species in the genus *Spea*, *S. multiplicata* is the basal species, with *S. bombifrons* as its most distantly related congener (Wiens & Titus 1991; García-París *et al.* 2003).

Larvae of both species exhibit trophic polyphenism: They develop either into an omnivore morph, which feeds mostly on organic detritus on the pond bottom, or a larger, morphologically distinct carnivore morph, which specializes on anostracan fairy shrimp (Pomeroy 1981; Pfennig 1990, 1992). The carnivore morph is induced by the ingestion of shrimp (Pomeroy 1981; Pfennig 1990). Moreover, both species grow better on shrimp (Pfennig & Murphy 2000), suggesting that it is the more nutritious resource.

In the San Simon Valley of SE AZ, the two species exhibit ecological character displacement in tadpole morph production (Pfennig & Murphy 2000, 2003; Pfennig *et al.* 2006). In ponds where each species occurs alone, both species produce similar, intermediate frequencies of each morph. However, in ponds where they co-occur, *S. multiplicata* produce almost entirely omnivores, whereas *S. bombifrons* produce almost entirely carnivores (Pfennig & Murphy 2000, 2003; Pfennig *et al.* 2006). Experiments reveal that this sympatric divergence in morph production has evolved because of interspecific resource competition (Pfennig & Murphy 2002; Pfennig *et al.* 2007).

Because *S. bombifrons* outcompetes *S. multiplicata* for the more nutritious resource (fairy shrimp), *S. bombifrons* can be deemed the winner of this competitive interaction. Indeed, character displacement appears to be costly for *S. multiplicata*: *S. multiplicata* are significantly smaller in body size in syntopy than in nearby allotopy (Pfennig & Pfennig 2005). This shift in body size likely reflects, at least in part, character displacement in tadpole morph production (Pfennig & Pfennig 2005). As noted above, *S. multiplicata* produce mostly omnivores in sympatric populations; omnivores are smaller at metamorphosis than carnivores and likely also mature at smaller size. Smaller body size, in turn, is associated with lower survival and fecundity (Pfennig & Pfennig 2005).

Methods

This study had two goals. First, we sought to determine which species more recently invaded the San Simon Valley of SE AZ (where character displacement has been documented). Second, we sought to identify the approximate ancestral range of the invader, *S. bombifrons*. This information was used to infer possible historical patterns of selection on *S. bombifrons* that may have predisposed this species to monopolize the superior resource.

Sampling

We collected adults and tadpoles of both species during summers 1999-2006 in SE AZ and SW NM (Fig. 3.1). Adults were collected at or near breeding aggregations; tadpoles were sampled from random sites throughout natural ponds using a hand-held dip net seven to 15 days posthatching. We sampled three types of ponds, which differed in their species composition: 1) ponds in which *S. multiplicata* was the only species of *Spea* present (pure *S. multiplicata* ponds; N=17); 2) ponds in which *S. bombifrons* was the only species of *Spea* present (pure *S. bombifrons* ponds; N=6); and 3) ponds in which both species of *Spea* were present (syntopic ponds; N=10; see Fig. 3.1 and Tables A.1 and A.2 for additional collection information). Within a few hours of collection, tadpoles were killed by immersion in a 0.1% aqueous solution of tricane methanesulfonate (MS 222), and preserved in 95% ethanol. We also obtained from museums and individual collectors additional *S. bombifrons* tissue samples from throughout their geographic range (Number of locations=38; Fig. 3.1, Table A.1).

DNA Extraction, Amplification, and Sequencing

We extracted genomic DNA from adult and tadpole tissues (Appendix). We then amplified and sequenced a 663 basepair portion of the cytochrome *b* (cyt *b*) gene from the mitochondrial genome (mtDNA; see Appendix). We sequenced an average of 15.5 *S. multiplicata* individuals from each of 27 locations (5-36 individuals per location; Table A.2), and an average of 6.4 *S. bombifrons* individuals from each of 54 locations (1-33 individuals per location; Table A.1).

Determining Order of Invasion

To determine which species invaded SE AZ more recently, we used three approaches. First, we calculated and compared haplotype and nucleotide diversities for the two species across the same region. Second, we examined patterns of isolation by distance in the two species. Finally, using a coalescent-based analysis, we estimated population growth in SE AZ populations of *S. bombifrons* and *S. multiplicata*.

Because it likely experienced a population bottleneck more recently as a result of colonization, we predicted that the more recent invader to this geographic region should exhibit lower genetic variation. Although numerous factors (e.g., selection, mutation rate, gene flow, demography) may affect levels of genetic variation in different species differentially, the fact that these two species experience similar ecological selection pressures and are similar in their phylogenetic position, generation times, and dispersal capabilities suggests that a cross-species comparison should provide useful information about differences in recent demographic history. We used ARLEQUIN 2.0 (Schneider *et al.*

2000) to calculate haplotype diversity and nucleotide diversity for each species. We then compared haplotype and nucleotide diversities for the two species over the entire region. We also calculated haplotype and nucleotide diversities for each species in each sampled pond separately (Tables A.1 and A.2) and compared the two species' mean diversity values by using non-parametric Wilcoxon Rank Sum tests. We employed non-parametric tests because the data did not meet parametric assumptions.

We tested both species for isolation by distance (IBD; Slatkin 1993), or a positive correlation between geographic distance and genetic distance. A signature of IBD should be evident for populations in migration-drift equilibrium. A non-equilibrium population, such as a recent invader, would not be expected to exhibit IBD, however (Slatkin 1993). To control for any differences between species in geographic spread of the samples, we tested for IBD only across ten syntopic ponds in the San Simon Valley (Fig. 3.1, Tables A.1 and A.2). We used Mantel tests in ARLEQUIN 2.0 to assess any correlation between population pairwise log-transformed geographic distance and population pairwise genetic distance (F_{ST}).

We predicted that a more recent invader should exhibit a higher rate of population growth. We used a coalescent-based Bayesian analysis, as implemented in LAMARC 2.1.2b (Kuhner 2006), to estimate Θ (= $2N_f\mu$, where N_f = effective number of females in the population, and μ = mutation rate per site per generation) and exponential growth rates (g, in units of μ^{-1}) for each of the two species in SE AZ. These parameters can be used to estimate the relative population sizes of *S. multiplicata* and *S. bombifrons* at a given time in the past (Wares & Cunningham 2001; Marko 2004; See Apppendix). For

each species, we sampled 100,000 genealogies with a sampling interval of 100 after discarding 10,000 genealogies as burn-in. We replicated these analyses three times.

Estimating S. bombifrons' Ancestral Range

To determine the ancestral range of *S. bombifrons*, we compared levels of genetic variation from populations across the range of the species. We predicted that the ancestral range should exhibit higher molecular diversity values than more newly colonized regions (Begun & Aquadro 1993; Hewitt 2000). Before we did this, we identified discrete populations to compare by examining hierarchical population structure using an AMOVA in ARLEQUIN 2.0. We grouped samples from each collection location into subpopulations, and then grouped the subpopulations together until the maximum amount of variation was explained by the groupings (Fig. 3.1, Table A.1). We then compared both haplotype and nucleotide diversities qualitatively among these regions.

Because range expansions often produce distinctive tree topologies (i.e., a star-burst pattern; Ball *et al.* 1988; Avise 2004), we estimated a phylogenetic tree to determine both whether *S. bombifrons* showed signatures of range expansion, and how widespread the expansion may have been. For comparison, we also included *S. multiplicata* samples from SE AZ. We estimated the phylogenetic relationships among the sampled cyt *b* haplotypes using a Bayesian analysis as implemented by MRBAYES 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). To root the tree, we included in the analysis three partial cyt *b* sequences from *Spea's* sister genus *Scaphiopus* (GenBank Accession Numbers AY236791-AY236793; García-París *et al.* 2003). We implemented the Hasegawa *et al.* (1985) model of DNA substitution with equal rates among sites

(HKY), which MODELTEST 3.6 (Posada & Crandall 1998) identified as the most likely model for our data. We performed two runs of the Bayesian analysis with four chains each, lasting 4.0 x 10^6 generations. From these runs, 80,002 trees were produced (40,001 for each run), of which 8000 were discarded as burn-in.

Results

Determining Order of Invasion

In comparing *S. multiplicata* and *S. bombifrons* from the region where they are undergoing character displacement, *S. bombifrons* showed lower overall haplotype and nucleotide diversities than *S. multiplicata* (mean (S.D.) haplotype diversity: 0.239 (0.040) vs. 0.543 (0.028), respectively; mean (S.D.) nucleotide diversity: 0.00038 (0.00047) vs. 0.00197 (0.00136), respectively). Indeed, *S. bombifrons* exhibited significantly lower haplotype ($W_{17,27} = 754$, $P = 0.0004$) and nucleotide diversities ($W_{17,27} = 786$, $P < 0.0001$) than *S. multiplicata*. These values are consistent with more recent colonization by *S. bombifrons*.

The two species exhibited different patterns of IBD across the ten syntopic ponds in SE AZ. While *S. multiplicata* exhibited a significant signature of IBD ($r = 0.48$, $P = 0.009$, based on 100,000 permutations), *S. bombifrons* showed no significant pattern of IBD ($r = 0.25$, $P = 0.15$, based on 100,000 permutations), again, suggesting that this species may have more recently invaded.

Spea bombifrons exhibited a significantly higher population growth rate than *S. multiplicata* (Table 3.1). This suggests that the *S. bombifrons* population in SE AZ is growing very quickly, as might be expected by a species that recently invaded.

Conversely, the population growth rate for *S. multiplicata* is not significantly different from zero, a value that indicates a stable population size. Moreover, the relative female effective population size 100,000 years ago for *S. bombifrons* is significantly smaller than for *S. multiplicata* (Table 3.1). This suggests that *S. bombifrons* in SE AZ have experienced a more recent population bottleneck, as would be expected from the more recent invader.

Estimating S. bombifrons' Ancestral Range

Our hierarchical population structure analysis revealed three discrete population groups across the range of *S. bombifrons* (Fig. 3.1; Table A.5): a Northern group, a Central group, and a Southwestern group. Of these three groups, the Central group, located in the southern Great Plains (Fig. 3.1), exhibited the highest haplotype and nucleotide diversities (Fig. 3.2). This suggests that the ancestral range of *S. bombifrons* was in the southern Great Plains.

The phylogenetic analysis suggests that *S. bombifrons* has likely undergone expansion throughout its entire geographical range. The clade as a whole forms a starburst pattern, exhibiting very little genetic differentiation or geographic structure (Fig. 3.3). This phylogeny also illustrates the greater degree of genetic differentiation in *S. multiplicata* from SE AZ compared to *S. bombifrons* (Fig. 3.3).

Discussion

Three independent lines of evidence implicate *S. bombifrons* (the winner) as the more recent invader into SE AZ, where character displacement is taking place. First, *S.*

bombifrons has lower haplotype and nucleotide diversity values than *S. multiplicata*. Reduced genetic diversity may indicate historically small population sizes or bottlenecks, characteristic of a colonization event. Second, patterns of isolation by distance suggest that *S. multiplicata* is at equilibrium, whereas *S. bombifrons* is not, possibly because it has undergone a recent range expansion. Third, while *S. multiplicata* from SE AZ have a stable, or at most a slowly growing, population, *S. bombifrons* from the same area have a rapidly growing population. Moreover, *S. bombifrons* exhibits signs of a more recent population bottleneck, perhaps due to a founding event. Fast population growth following a bottleneck may characterize recent invaders. These growth rates are also consistent with a previous study that found a recent increase in the relative frequency of *S. bombifrons* at breeding aggregations in SE AZ (Pfennig 2003).

Although multiple lines of evidence implicate *S. bombifrons* as the more recent invader into SE AZ, our data do not allow us to entirely rule out an alternative hypothesis: that *S. bombifrons* was resident in SE AZ and underwent a demographic expansion after *S. multiplicata* invaded. In this alternative scenario, however, the impact of *S. bombifrons* on the competitor, *S. multiplicata,* is nearly equivalent to what it would be were *S. bombifrons* the invader. For character displacement to occur, population sizes of competing species must be large enough to deplete shared resources, generating interspecific competition (Grant & Grant 2006). Either scenario would therefore have produced new selective pressures favoring interspecific divergence in resource use and associated traits. Moreover, both scenarios are consistent with the idea that species able to very quickly increase population size in the face of competition, as invasive species do, might tend to win in character displacement.

Two lines of evidence indicate that *S. bombifrons* underwent a widespread range expansion out of its ancestral range in the southern Great Plains. First, high levels of genetic variation in the southern Great Plains (i.e., the Central group, Figs. 3.1, 3.2) suggest that this region is likely the ancestral range for *S. bombifrons*. Second, the haplotype tree (Fig. 3.3) shows a starburst-shaped *S. bombifrons* clade with a widespread haplotype (haplotype 1) and an excess of rare haplotypes (Table A.3). This topology is consistent with recent expansion throughout the entire range, and suggests that populations have been relatively recently connected genetically (Ball *et al.* 1988; Avise 2004).

Much of *S. bombifrons*' expansion from their ancestral range northward is likely the result of post-Pleistocene expansion after the glaciers receded. Recent southward expansion may have been driven, in part, by anthropogenic changes to the environment. Because *S. bombifrons* tadpoles develop more slowly than do *S. multiplicata* tadpoles (Pfennig & Simovich 2002), *S. bombifrons* was probably unable to breed in the highly ephemeral ponds that historically characterized much of SE AZ. Beginning in the 1880s, however, ranchers began to excavate longer lasting "cattle tanks" (Gehlbach 1981; Bock & Bock 2000), which now serve as *Spea*'s primary breeding sites (A. Rice & D. Pfennig, pers. observ.). Consequently, slower developing species (such as *S. bombifrons*) that normally do not live in arid regions occur in SE AZ. Other possible causes for the southward expansion remain unclear.

Given that *S. bombifrons* appears to be the invader into SE AZ, we also sought to understand why this species, as opposed to *S. multiplicata*, won during character displacement. For at least two reasons, historical patterns of selection in the ancestral

range may have pre-adapted *S. bombifrons* to monopolize the superior shrimp resource. First, because *Spea* follows Bergmann's Rule (adult body size increases with increasing latitude; R. Martin & D. Pfennig; unpubl. data), *S. bombifrons* invading from the north were likely larger than the resident *S. multiplicata* (as shown in Fig. 3.1, *S. multiplicata* has a more southerly distribution; thus, allopatric *S. multiplicata* are smaller than allopatric *S. bombifrons*). Because larger females produce larger tadpoles (R. Martin & D. Pfennig, unpubl. data), which, in turn, are better predators of shrimp (Frankino & Pfennig 2001), *S. bombifrons* may have been predisposed to monopolize the superior shrimp resource. Second, the lineages of *S. bombifrons* that invaded SE AZ likely had an historical association with *S. multiplicata*; they would have encountered the northeastern edge of *S. multiplicata*'s range in an earlier stage of the expansion from their ancestral range (Fig. 3.1). By contrast, *S. multiplicata* in SE AZ would not have previously encountered *S. bombifrons*. Consequently, *S. bombifrons* had likely experienced prolonged selection to outcompete *S. multiplicata* for the superior shrimp resource. Generally, why one species wins during character displacement may depend on a variety of factors, including, but not limited to, historical patterns of selection on behavior or morphology that pre-adapt individuals for competitive interactions with naïve interspecifics.

Do invaders generally win during character displacement? We cannot answer this question definitely because the fitness consequences of character displacement are not known in most other systems that have undergone character displacement. In at least one other case, however, the invader appears to have won. The medium ground finch, *Geospiza fortis*, was already present on the Galápagos island of Daphne Major when the

large ground finch, *G. magnirostris,* invaded. Following this invasion, the two species underwent character displacement in resource use and beak morphology that enabled the invader to monopolize the more nutritious seed resource (Grant & Grant 2006). Thus, as in spadefoots, the invader has apparently won during character displacement in *Geospiza* finches. Further research into additional cases of character displacement is necessary to determine if invaders generally win, however. Because successful invaders may often be superior competitors (Sakai *et al.* 2001; Vila & Weiner 2004; Rehage *et al.* 2005), invasive species may generally be more likely to win during character displacement. Moreover, we may only detect character displacement when the invader monopolizes the more profitable resource; because population sizes should be smaller for recent invaders in general, any invaders that fail to monopolize the more profitable resource are more likely to go extinct.

In sum, population genetic, phylogenetic, and phylogeographic analyses, when combined with information about fitness trade-offs, can shed light on the outcome of character displacement. Ultimately, this historical perspective may help us to understand whether invaders generally win during character displacement, and, if so, why.

Table 3.1. Estimates of Θ and *g* for *S. multiplicata* and *S. bombifrons* in southeast Arizona. The confidence intervals presented are 95% highest posterior density credible regions (HPD). Relative N_f was calculated using the point estimate for Θ and the endpoints of the 95% HPD for *g*, a generation time of 2 years, and a mutation rate of 4.0×10^{-9} substitutions per site per generation (Tan & Wake 1995). Any discrepancy between this rate and the actual mutation rate for cyt *b* in *Spea* will only affect the time estimate; it will not affect the comparison between the two species. Likewise, while estimates of *g* tend to be biased upward when based on one gene (Kuhner *et al.* 1998), the relative estimates for the two species should not be affected. Details of the calculations performed can be found in the Appendix.

Species	**Θ (95% HPD)**	***g* (95% HPD)**	**Relative N_f 100,000 years ago**
S. multiplicata	0.007 (0.0042-0.0123)	12.58 (-413.2-602.4)	0.99 (0.89-1.09)
S. bombifrons	0.004 (0.0011-0.0159)	10,510 (1803-15,100)	0.12 (0.05-0.7)

Figure Legends

Figure 3.1. Potential geographic ranges and sampling locations of *Spea bombifrons* and *S. multiplicata* (see Appendix for more detailed location information). The solid white line surrounds the range of *S. bombifrons*; the dashed white line indicates the range of *S. multiplicata* (ranges based on Stebbins 2003). The inset shows the San Simon Valley (center) and the immediately surrounding valleys in southeastern Arizona and southwestern New Mexico. Symbols represent sampling locations: solid white symbols are *S. bombifrons* sampling locations, solid black symbols are *S. multiplicata* sampling locations, and white squares hatched with black lines are sampling locations where both species were present ("syntopy"). For closely clumped sampling locations, one symbol may be used to represent multiple locations. More than one individual may have been collected at each sampling location (see Appendix). Symbol shapes designate the geographic group to which each *S. bombifrons* sampling location was assigned (see Methods): white diamonds—Northern group; white circles—Central group; white squares (solid and hatched)—Southwestern group.

Figure 3.2. Mean genetic diversity measures for three populations across the geographic range of *S. bombifrons*. From left to right on the x-axis, latitude decreases (see Fig. 3.1). Top, mean haplotype diversity ± standard deviation. Bottom, mean nucleotide diversity ± standard deviation.

Figure 3.3. Phylogenetic relationships among 47 unique *Spea bombifrons* and *S. multiplicata* cytochrome *b* haplotypes (663 bp). The tree shown is the majority-rule

consensus cladogram based on a Bayesian analysis. Clade support values (boldface proportions) at each node are Bayesian posterior probabilities. Each branch is labeled with the branch length, in units of substitutions per site. Haplotype numbers at the tips of the cladogram follow the numbering scheme from Tables A.3 and A.4. The symbols at the end of each branch indicate the location(s) where each haplotype was found, corresponding with the geographic groupings in Figure 3.1.

Figure 3.1

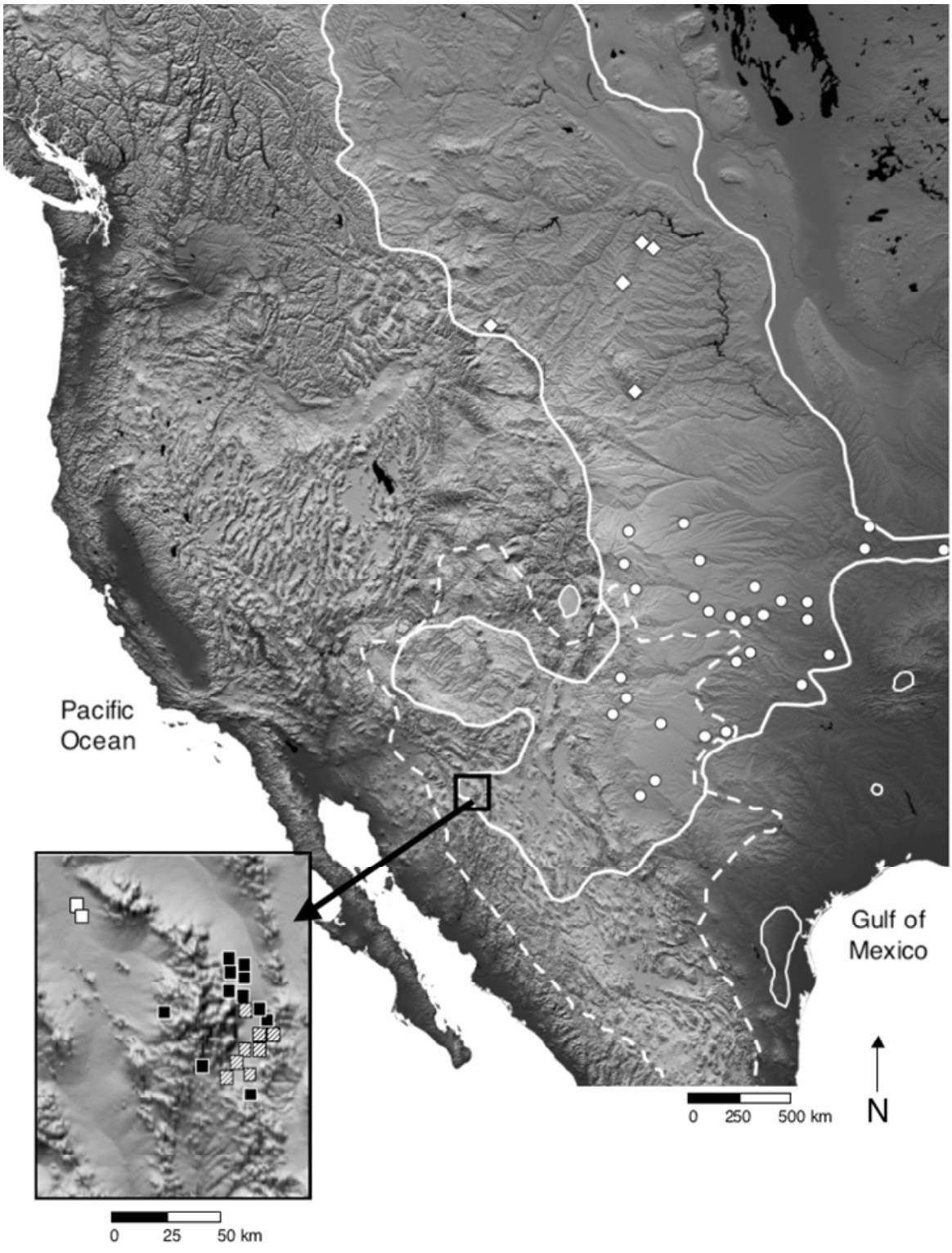


Figure 3.2

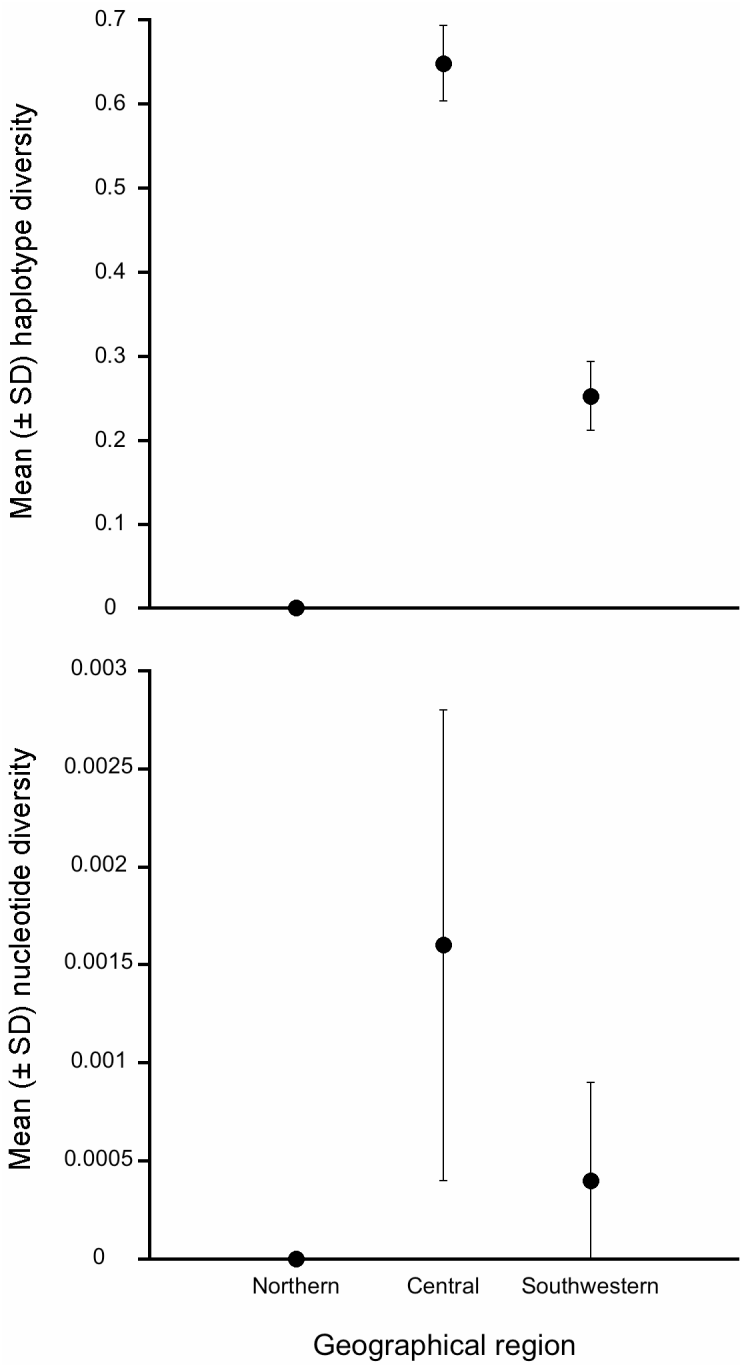
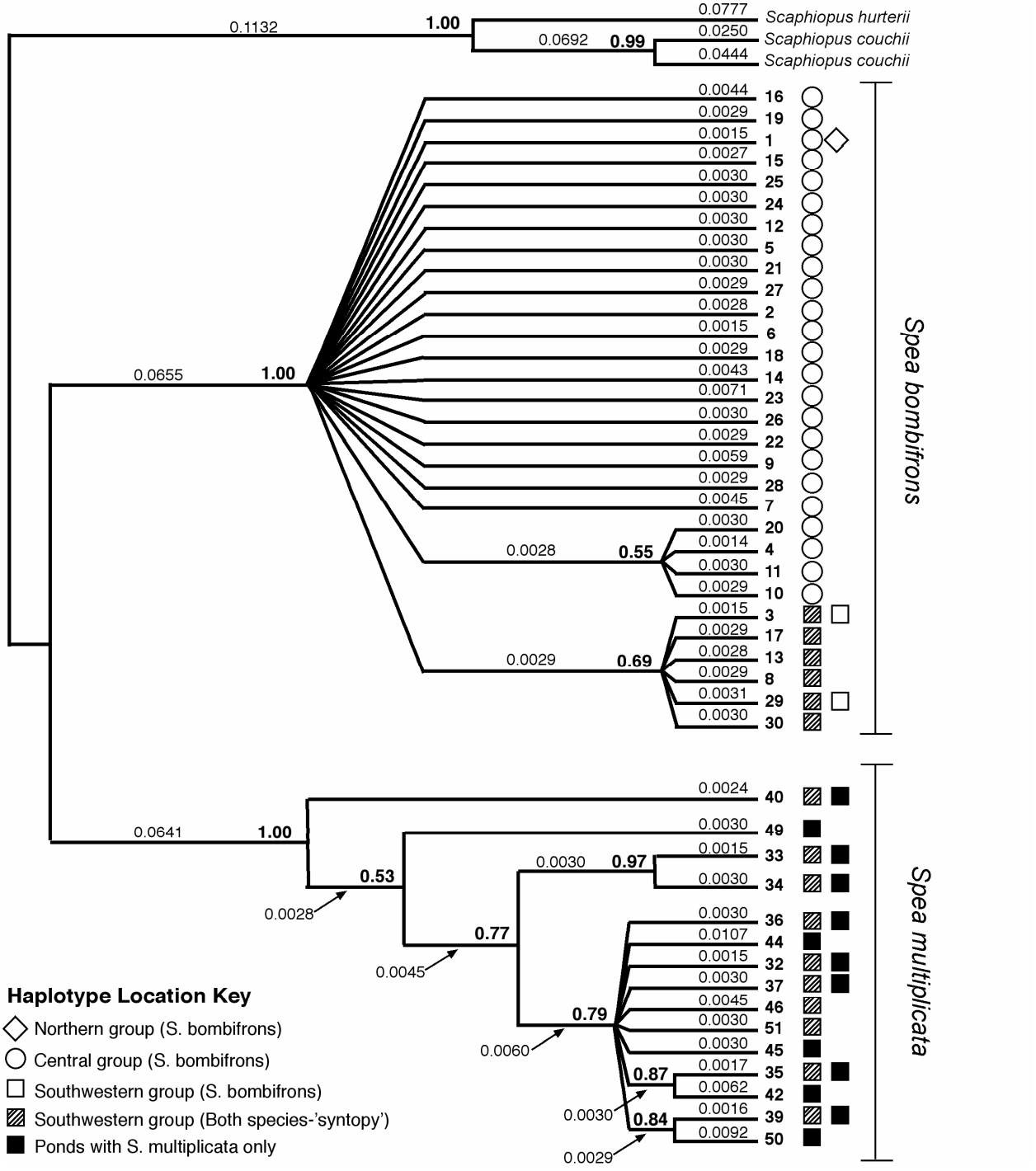

Mean (± SD) haplotype diversity
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Mean (± SD) nucleotide diversity
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
Northern
Central
Southwestern
Geographical region

Figure 3.3


0.1132
1.00
0.0692
0.99
0.0777
Scaphiopus hurterii
0.0250
Scaphiopus couchii
0.0444
Scaphiopus couchii
0.0655
1.00
0.0044 16
0.0029 19
0.0015 1
0.0027 15
0.0030 25
0.0030 24
0.0030 12
0.0030 5
0.0030 21
0.0029 27
0.0028 2
0.0015 6
0.0029 18
0.0043 14
0.0071 23
0.0030 26
0.0029 22
0.0059 9
0.0029 28
0.0045 7
0.0028
0.55
0.0030 20
0.0014 4
0.0030 11
0.0029 10
0.0029
0.69
0.0015 3
0.0029 17
0.0028 13
0.0029 8
0.0031 29
0.0030 30
Spea bombifrons
0.0641
1.00
0.0024 40
0.0030 49
0.0030
0.97
0.0015 33
0.0030 34
0.53
0.0028
0.77
0.0045
0.0030 36
0.0107 44
0.0015 32
0.0030 37
0.0045 46
0.79
0.0030 51
0.0060
0.0030 45
0.0017 35
0.87
0.0062 42
0.0030
0.0016 39
0.84
0.0092 50
0.0029
Spea multiplicata
Haplotype Location Key
Northern group (S. bombifrons)
Central group (S. bombifrons)
Southwestern group (S. bombifrons)
Southwestern group (Both species-'syntopy')
Ponds with S. multiplicata only

CHAPTER IV

EVIDENCE FOR INDEPENDENT DIVERGENCE IN A SPECIES UNDERGOING CHARACTER DISPLACEMENT

Summary

When documenting ecological character displacement — trait evolution stemming from selection to lessen resource competition between species — chance must be ruled out as an explanation for trait divergence. This is often done by measuring resource-use phenotypes in multiple sympatric and allopatric populations, and demonstrating that species consistently differ in sympatry but not in allopatry. Often, however, no evidence is presented to establish the evolutionary independence of the different populations used in such analyses. If populations are not independent, then character displacement has not been replicated, and the evidence against chance is weakened. Here, we use genetic and morphological data to test for independent displacement in multiple sympatric populations of spadefoot toads, *Spea multiplicata*, which previous research has suggested are undergoing ecological character displacement. We found that most sympatric populations have experienced independent displacement. Tadpoles in many of the most closely related populations exhibit very different trophic morphology. Moreover, more of the variation in morphology among populations is explained by differences in competitive environment than by differences in population relatedness. However, we did find evidence supporting the non-independence of some sympatric populations. Our data therefore underscore the importance of using genetic data to establish the evolutionary independence of populations when documenting ecological character displacement.

Introduction

Ecological character displacement, or trait evolution stemming from selection to lessen resource competition between species, has long been considered an important cause of adaptive radiations and species coexistence (reviewed in Schluter 2000b). When two species compete for limited resources, individuals that are most similar to the other species in resource use and associated traits should experience enhanced competition. Consequently, these individuals suffer reduced fitness (Schluter 1994; Pfennig *et al.* 2007), and divergent directional selection favors those individuals of each species that are most unlike the other species. Over time, this selection may lead to character displacement in resource use and associated traits.

The process of ecological character displacement often produces a distinctive pattern whereby closely related species are recognizably different in resource-use traits in sympatry, even though they may be similar in such traits in allopatry (but see Goldberg & Lande 2006). However, because numerous other evolutionary processes may produce such a pattern (e.g., Grant 1972, Arthur 1982), six criteria for demonstrating ecological character displacement using observational data have been identified (Schluter & McPhail 1992; Taper & Case 1992). One of these criteria requires that chance be ruled out as an explanation for the pattern of divergent traits in sympatry.

One way to demonstrate that chance has not played a major role in generating divergence is to document that divergence has occurred repeatedly in multiple independent cases (Schluter & McPhail 1993; Schluter 2000a). Such a scenario instead strongly implicates natural selection as the agent of divergence (Schluter & Nagel 1995; Rundle *et al.* 2000; Johannesson 2001; Nosil *et al.* 2002; Langerhans *et al.* 2007;

Quesada *et al.* 2007). In many putative cases of ecological character displacement, chance has been ruled out by measuring the magnitude of phenotypic displacement in multiple populations, and documenting that the displacement is greater than some null expectation (e.g., Fenchel 1975; Dunham *et al.* 1979; Adams & Rohlf 2000; Pfennig & Murphy 2000). It is possible in such cases, however, that resource competition has lead to interspecific divergence in one population, and then those phenotypes spread as a result of gene flow to other populations. If this were the case, then each population would not be an independent replicate of divergence, and the evidence against chance as the agent of divergence becomes much weaker. To address this issue, genetic data can be useful to establish the relationships among populations of each species to determine whether divergence really has occurred independently (Schluter 2000a; Marko 2005). Here, we use genetic and morphological data to test for independent divergence of trophic characters in a species that is undergoing character displacement—the Mexican spadefoot toad, *Spea multiplicata*.

Study System

Mexican spadefoot toads, *Spea multiplicata*, and Plains spadefoot toads, *S. bombifrons*, co-occur in the San Simon Valley of southeastern Arizona and southwestern New Mexico (Fig. 4.1). In this region, both species co-occur below 1350 m in elevation (hereafter termed "sympatry"), while at higher elevations, only *S. multiplicata* is present (hereafter termed "allopatry"; Pfennig *et al.* 2006).

Larvae of both species exhibit trophic polyphenism. They may develop into either a small-headed omnivore morph, which feeds mostly on organic detritus on the pond

bottom, or a large-headed, morphologically distinct carnivore morph, which specializes on anostracan fairy shrimp in the water column (Pomeroy 1981; Pfennig 1990, 1992a). Carnivores have enlarged jaw muscles (the orbitohyoideus (OH) and interhyoideus (IH) muscles) and serrated beaks (Pomeroy 1981; Pfennig 1992b), which likely improve foraging ability on fairy shrimp (Satel & Wassersug 1981; Ruibal & Thomas 1988; Pfennig 1992b; R. A. Martin, unpubl. data). The carnivore morph is induced by the ingestion of shrimp (Pomeroy 1981; Pfennig 1990).

These two species appear to exhibit ecological character displacement in tadpole morph production (Pfennig & Murphy 2000, 2003; Pfennig *et al.* 2006, 2007). In ponds where each species occurs alone, both species produce similar, intermediate frequencies of each morph. However, in ponds where they co-occur, *S. multiplicata* produce almost entirely omnivores, whereas *S. bombifrons* produce almost entirely carnivores (Pfennig & Murphy 2000, 2003; Pfennig *et al.* 2006). Experiments reveal that this sympatric divergence in morph production has likely evolved because of interspecific resource competition (Pfennig & Murphy 2002; Pfennig *et al.* 2007).

Although morph production in *Spea* is mediated in part by phenotypic plasticity, it involves a heritable component as well. When raised under common conditions, certain sibships show a greater propensity than others to produce the carnivore morph (Pfennig & Frankino 1997; Pfennig 1999). Indeed, *S. multiplicata* tadpoles with parents collected from where *S. bombifrons* is prevalent are less likely to become carnivores than tadpoles produced by parents collected from where *S. bombifrons* is absent (Pfennig & Murphy 2000; 2002). This suggests that *S. multiplicata* from sympatric and allopatric ponds in the

San Simon Valley of southeastern Arizona have undergone evolutionary divergence in propensity to produce the carnivore morph.

Specific Predictions

In this study, we focused on divergence in *S. multiplicata* tadpole morphology between individuals from populations in allopatry and individuals from populations in sympatry. If competition with *S. bombifrons* displaced *S. multiplicata* tadpoles to the omnivore phenotype only once, with gene flow subsequently responsible for spreading this adaptive phenotype to other *S. multiplicata* populations in sympatry, we predicted that *S. multiplicata* populations more similar in morphology should also be more similar genetically. This prediction specifically applies to populations in sympatry; populations in allopatry, which presumably exhibit the ancestral phenotype, may or may not be similar genetically. Therefore, under this scenario, we would expect to see a population phylogeny similar to figure 4.2a. In contrast, if character displacement evolved independently multiple times, with *S. bombifrons* displacing *S. multiplicata* to the omnivore phenotype repeatedly, then we would expect to find no clear relationship among genetic similarity of sympatric populations and mean trophic morphology (Fig. 4.2b). Moreover, we would expect to find that differences among populations in competitive environment (presence or absence of *S. bombifrons*) should more strongly predict differences among populations in trophic morphology than would genetic distance among populations.

Methods

In order to assess whether character displacement occurred independently in multiple *S. multiplicata* populations, we asked three questions. First, how does tadpole trophic morphology vary among populations in the San Simon Valley? Second, are populations with more similar morphology also more closely related genetically? Third, do differences in competitive environment or differences in relatedness among populations better account for the variation in trophic morphology?

Sampling

We collected *S. multiplicata* tadpoles during summers 1999-2004 in southeastern AZ and southwestern NM. Tadpoles were sampled seven to 15 days posthatching from random sites throughout natural, temporary ponds using a hand-held dip net. We sampled ten allopatric ponds and seven sympatric ponds (Fig. 4.1; Table 4.1). Within a few hours of collection, tadpoles were killed by immersion in a 0.1% aqueous solution of tricane methanesulfonate (MS 222) and preserved in 95% ethanol. For each pond site, we used Google Earth version 4.2.0198.2451 (beta) to determine latitudinal and longitudinal coordinates. The geographic coordinates were used to calculate geographic distance between each pair of pond sites using the great circle formula as implemented by the GPS WAYPOINT REGISTER'S distance calculator (http://www.gpswaypoints.co.za/Downloads/distcalc.xls).

DNA Extraction, Amplification, Sequencing, and Genotyping

We used two procedures for extracting DNA. For tadpoles collected from 1999-2001, we extracted genomic DNA using the DNeasy Blood & Tissue Kit (QIAGEN), following

the manufacturer's protocol for extractions from animal tissue samples. For tadpoles collected from 2002-2004, we incubated tissues overnight with Proteinase K (QIAGEN), extracted DNA using a saturated NaCl solution, and precipitated and washed the DNA using ethanol.

We amplified and sequenced a portion of the cytochrome *b* (cyt *b*) gene from the mitochondrial genome (mtDNA). We used a forward primer designed from an *S. multiplicata* sequence (SCB1-F; 5'- TCCCAACCCCATCTAACATC-3') and a reverse primer designed from a *Xenopus laevis* sequence (XCB2-R; 5'-GAGGGCTAAGATTAGGATGGATA-3'). We carried out 40 cycles of the polymerase chain reaction on the MJ Research PTC-200 DNA Engine thermal cycler using the following profile: 94 ºC for 30 s; 50 ºC for 30 s; 72 ºC for 90 s. The amplification products were purified using ExoSAP-IT® (USB). After purification, we submitted the amplification products to the UNC-Chapel Hill Genome Analysis Facility for direct sequencing on an ABI 3730 Genetic Analyzer. We obtained cyt *b* sequences from a total of 275 individuals (8-28 individuals per pond; Table 4.1). Using SEQUENCHER 4.5 (GeneCodes), we assembled the sequence chromatograms for each sample into contigs and proofread the sequences. We then aligned all the sequences using CLUSTALX 1.83 (Thompson *et al.* 1997), and trimmed them to a length of 663 base pairs using MACCLADE 4.08 (Maddison & Maddison 1989). We used both PAUP* 4.0b10 (Swofford 2002) and MACCLADE 4.08 to group the sequences into unique haplotypes (These cyt *b* sequences were previously published in Rice & Pfennig 2008; GenBank accession nos. EU285643-EU285652, EU285654, EU285657).

For eight to ten individuals per pond (Table 4.1), we amplified eight previously published microsatellite loci (three di-nucleotide loci: *Sm1*, *Sm4*, *Sm23*; two tri-nucleotide loci: *Sb15*, *Sb28*; three tetra-nucleotide loci: *Sm14*, *Sm20*, *Sm25*; GenBank Accession Numbers EU285444-EU285445, EU285450-EU285452, EU285454-EU285456) using the published protocols (Rice *et al.* in press). We submitted the amplified products to the UNC-Chapel Hill Genome Analysis Facility for genotyping on an ABI 3730 Genetic Analyzer. Peaks were scored based on an internal size standard (GeneScan™-500 LIZ®; Applied Biosystems) using GeneMapper v3.7 (Applied Biosystems).

Hardy-Weinberg equilibrium and linkage disequilibrium at microsatellite loci

For the microsatellite loci, we used the probability test in GENEPOP 4.0.6 (Rousset 2008) to test each locus for Hardy-Weinberg Equilibrium (HWE). Statistical significance was estimated using the Markov chain method, with 10,000 dememorizations, 1000 batches, and 10,000 iterations per batch. Because tests for HWE were performed for each pond-locus combination, we adjusted the α-value for each locus using sequential Bonferroni corrections (Rice 1989). We also tested for linkage disequilibrium between all pairs of loci across all ponds using Fisher's global test in GENEPOP 4.0.6 (Rousset 2008).

Variation in trophic morphology

Following the methods of Pfennig *et al.* (2007), we measured three diagnostic trophic characters on a total of 458 tadpoles (11-86 individuals per pond; Table 4.2): 1) width of the orbitohyoideus (OH) muscle; 2) width of the interhyoideus (IH) muscle; and 3) shape

of the keratinized mouthparts (MP). We also measured the body size of each tadpole (snout-vent length; SVL). In order to standardize the OH and IH measurements for body size, we regressed log OH and log IH on log SVL and used the residuals. Using a principal component (PC) analysis, we then combined the standardized OH and IH measurements with the MP score into a multivariate shape variable, the "morphological index" (Pfennig *et al.* 2006, 2007; Pfennig & Rice 2007). We used PC1, which explained 53.4% of the variance in the three characters, as the morphological index. Larger values of the morphological index indicated a more carnivore-like morphology, while smaller values were consistent with a more omnivore-like morphology.

We next characterized the *S. multiplicata* tadpole morphology in each of our ponds. Because of the ecological character displacement with *S. bombifrons*, we predicted that tadpoles from allopatric ponds should generally have higher morphological index scores than tadpoles from sympatric ponds (Pfennig *et al.* 2006). Because tadpoles within each pond are not independent replicates, we used the mean morphological index score from each pond for our comparison. Because we had outliers in our data, we used a non-parametric Wilcoxon Rank Sum test to compare the morphological index scores of sympatric versus allopatric ponds. Then, to visualize the variation in trophic morphology across all 17 ponds, we used JMP 5.1.2 (SAS Institute, Inc.) to create a dendrogram based on Ward's minimum variance clustering method (Østbye *et al.* 2006).

Determining population relatedness

To determine whether genetically similar populations share similar morphologies, which would suggest that character displacement had not evolved independently in each

population, we constructed a neighbor-joining tree for the 17 populations based on the eight microsatellite loci. We used MICROSATELLITE ANALYZER 4.05 (MSA; Dieringer & Schlötterer 2003) to generate 2500 bootstrap replicate matrices of distance measures among all pond pairs. We used Nei's D_A to construct the tree because this distance measure has been shown to be superior to F_{ST} for obtaining the correct tree topology in microsatellite neighbor-joining trees (Takezaki & Nei 2008). We then used the FITCH program in the PHYLIP 3.67 package (Felsenstein 1989, 2007) to generate 2500 neighbor-joining trees using Nei's D_A distances. We used CONSENSE in PHYLIP 3.67 (Felsenstein 1989, 2007) to generate an extended Majority Rule consensus tree from the 2500 neighbor-joining trees. To determine whether character displacement evolved independently in multiple populations, we mapped mean *S. multiplicata* tadpole morphology for each pond onto the tree.

Relative contributions of genetics and competitive environment on morphology

Next, we asked whether the variation among ponds in tadpole morphology could best be explained by genetic relationships among the ponds or the competitive environment of each pond. To address this question, we ran Mantel and partial Mantel tests with ARLEQUIN 3.11 (Schneider *et al.* 2000). Mantel (Mantel 1967) and partial Mantel tests (Smouse *et al.* 1986) assess correlations (or partial correlations) among distance matrices. They have frequently been used to disentangle the contributions of genetic relatedness and ecology to intraspecific morphological evolution (Thorpe *et al.* 1995; Thorpe 1996; Langerhans *et al.* 2007). We generated a dependent matrix of differences in the morphological index between all pond pairs by taking the absolute value of the difference

between each pond's mean morphological index score. A larger value in this matrix indicated that two ponds were very different in morphology. We then generated two predictor matrices. The first predictor matrix described genetic relationships among all pond pairs. For the microsatellite data, we used either pairwise F_{ST} values or Nei's D_A distances, both generated with MSA 4.05 (Dieringer & Schlötterer 2003). For the cyt *b* data, we used pairwise F_{ST} values calculated with ARLEQUIN 3.11 (Schneider *et al.* 2000). The second predictor matrix was categorical, and described differences in competitive environment between all pond pairs (i.e., 0=both ponds either lacked or contained the competitor, *S. bombifrons*; 1=one pond lacked *S. bombifrons* while one pond contained *S. bombifrons*). To estimate significance, we used 100,000 permutations of the data.

Results

Hardy-Weinberg equilibrium and linkage disequilibrium at microsatellite loci

Most pond-loci combinations did not show significant ($P < 0.003$) departure from Hardy-Weinberg equilibrium (HWE). In fact, only two loci, *Sm4* and *Sb15*, departed from HWE in more than one pond (*Sm4* departed from HWE in six out of 17 ponds; *Sb15* departed from HWE in 13 out of 17 ponds). None of the loci were in linkage disequilibrium ($P > 0.37$ for all loci pairs across all ponds).

Variation in trophic morphology

As we predicted, in general, allopatric populations exhibited more carnivore-like morphology (mean morphological index ± SEM; 0.243 ± 0.167) than sympatric populations (-0.604 ± 0.339; $W_{7,10}= 41$, $P = 0.032$). Likewise, the dendrogram showed

that most of the more omnivore-like populations occurred in sympatry, while most of the more carnivore-like populations were in allopatry (Fig. 4.3). Notable exceptions to this pattern were BP and JA. BP, which is an allopatric pond, showed more omnivore-like morphology than many of the sympatric ponds, while JA, a sympatric pond, showed more carnivore-like morphology than any other pond (Table 4.2, Fig. 4.3). Overall, allopatric ponds tended to be more carnivore-like, while sympatric ponds were more omnivore-like.

Determining pond relatedness

The neighbor-joining tree indicated that some sympatric *S. multiplicata* populations have evolved the displaced omnivore-like morphology independently (Fig. 4.4). This is evident by the multiple instances where closely related populations do not share similar trophic morphology, including JC and FT, and AP and the PC/YW clade (Fig. 4.4). However, the SD/SH/SC clade, which shows closely related populations sharing similar trophic morphology, also supports a role for gene flow in spreading the omnivore-like morphology (Fig. 4.4). Our results are therefore intermediate between the two predicted extremes (Fig. 4.2). This suggests that not only were multiple populations of *S. multiplicata* displaced to the omnivore phenotype independently, but that gene flow has also been responsible for the presence of this adaptive phenotype in several populations.

Relative contributions of genetics and competitive environment on morphology

Ponds variation in tadpole morphology was best explained by differences in the competitive environment. Without controlling for genetics, the competitive environment

matrix was significantly correlated with the morphological matrix (r = 0.43, *P* = 0.002). On the other hand, neither of the genetic distance matrices was correlated with the morphological matrix (Nei's D_A: r = 0.13, *P* = 0.13; Microsatellite F_{ST}: r = 0.04, *P* = 0.35; Cyt *b* F_{ST}: r = -0.04, *P* = 0.56). The results were the same when we used partial Mantel tests to control for the opposite predictor matrix. Thus, differences in competitive environment have a greater effect on differences in tadpole morphology than genetic relationships among populations. This is more consistent with repeated independent evolution of character displacement versus a single displacement with subsequent spreading of the adaptive phenotype by gene flow.

Discussion

In the spadefoot toad species *S. multiplicata*, which has undergone ecological character displacement, we used morphological and genetic data to ask whether populations in sympatry with a competitor, *S. bombifrons*, have undergone independent divergence in trophic morphology. Two lines of evidence suggest that multiple sympatric populations of *S. multiplicata* have experienced independent displacement to the omnivore phenotype. First, the neighbor-joining population tree indicates that some of the most closely related populations exhibit very divergent trophic morphology (Fig. 4.4). However, this tree is intermediate between the two predicted trees (Fig. 4.2), which indicates that some populations have been independently displaced to the omnivore phenotype, while others have likely gained this morphology as the result of gene flow from nearby sympatric populations. Second, in accord with the neighbor-joining tree, the matrix correlations are more consistent with multiple independent displacements to the

omnivore phenotype in sympatry: More variation in trophic morphology among populations is explained by differences in competitive environment than by differences in genetic distance. This study therefore supports a scenario in which ecological character displacement between *S. multiplicata* and *S. bombifrons* in southeastern Arizona and southwestern New Mexico has occurred repeatedly in independent populations (Langerhans *et al.* 2007). Such a result strongly implicates natural selection as the agent of divergence between allopatric and sympatric populations of *S. multiplicata*.

Although our results support the scenario outlined above, it should be noted that the bootstrap support for our neighbor-joining tree is very low. Given that the tree is depicting intraspecific relationships, this is not surprising. Low bootstrap support suggests that the relationships among the populations are not well resolved. Therefore, any conclusions that we draw from such a tree may not be robust. Analyses that are more appropriate for intraspecific comparisons are available, however, such as intraspecific independent contrasts (Felsenstein 2002), spatial autocorrelation (Marko 2005), and Mantel and partial Mantel tests (Mantel 1967; Smouse *et al.* 1986; Thorpe *et al.* 1995). In support of our overall conclusions, our Mantel test results did agree with the results from our neighbor-joining tree.

The strength of the correlation between variation in trophic morphology and differences in competitive environment may actually be stronger than what we found here. Pfennig & Murphy (2002) demonstrated that sympatric ponds vary in the relative frequency of the two species. Because we used a categorical variable to represent differences in the competitive environment between populations, we were unable to capture that variation and relate it to differences in trophic morphology. A stronger

correlation would make an even more convincing case for the importance of natural selection as the mechanism behind morphological divergence between sympatric and allopatric populations of *S. multiplicata*.

Of the six criteria necessary for demonstrating ecological character displacement (Schluter & McPhail 1992; Taper & Case 1992), the criterion that has been met by more studies than any other is ruling out chance as an explanation for exaggerated divergence between species in sympatry (Schluter 2000a). This usually requires replicated divergence in numerous populations. Most studies, however, do not use measures of genetic distance or gene flow to determine whether or not populations are actually independent (Schluter 2000a). Putative cases of character displacement in which such an approach has been used include the vine *Dalechampia scandens* (Hansen *et al.* 2000) and the gastropods *Nucella ostrina* and *N. emarginata* (Marko 2005). Using distance between populations as a proxy for relatedness, Hansen *et al.* (2000) developed a model for an intraspecific comparative analysis of character displacement. They found evidence that *D. scandens* blossom traits have locally adapted to the presence of competitors for the same set of pollinators (Hansen *et al.* 2000). However, because the presence or absence of competitors only explained up to 20% of the variation in blossom traits, they suggested that additional selective factors may also influence blossom morphology. In the gastropods *N. ostrina* and *N. emarginata*, Marko (2005) used a spatial autocorrelation analysis on genetic and morphological data to determine whether the divergent shell shapes and ornamentation exhibited by the two species in sympatric populations represent independent, replicated divergence. In *N. emarginata*, replicated independent divergence in sympatric populations was supported, while gene flow appeared to play a

more important role in the shell shape and ornamentation shifts of *N. ostrina* (Marko 2005). Our results for *S. multiplicata* are therefore consistent with these previous comparative studies of character displacement, suggesting that both local adaptation to competitors and population history may be important in generating patterns of exaggerated trait divergence in sympatry.

Our data also have a bearing on helping to distinguish by which evolutionary route character displacement arose in *Spea*. Character displacement may arise through two possible routes: *in situ* evolution of novel phenotypes (ISE) or sorting of pre-existing variation (sorting; Rice & Pfennig 2007). With ISE, divergent traits arise and spread in sympatry following first contact with the competitor species. On the other hand, with sorting, the source of and initial selection for divergent traits is in allopatry, before the species come into contact (Rice & Pfennig 2007). These two routes are not mutually exclusive, but distinguishing between them can be important for understanding the causes and consequences of character displacement (Rice & Pfennig 2007). When population history or relatedness is found to play an important role in sympatric trait divergence, sorting may be the primary route by which character displacement evolved. In contrast, when multiple populations show independent divergence in sympatry, as our results show for *S. multiplicata*, character displacement may have more likely resulted from ISE (Rice & Pfennig 2007). Yet, because we found some evidence supporting the non-independence of some sympatric populations, our data also suggest that sorting may have also played a role in mediating character displacement in *S. multiplicata*.

Finally, our results support previous conclusions from this system about the role of phenotypic plasticity in character displacement (Pfennig & Murphy 2002; Pfennig *et al.*

2006). Specifically, character displacement may not occur solely as the result of genetic differences between populations in sympatry and allopatry ("canalized character displacement" *sensu* Pfennig & Murphy 2002). Rather, phenotypic plasticity may also play an important role in allowing a species to respond adaptively to the presence of a competitor ("facultative character displacement" *sensu* Pfennig & Murphy 2002; Pfennig *et al.* 2006). *Spea* tadpoles exhibit trophic polyphenism, and can therefore respond to the presence of a competitor through facultative changes in trophic morphology (Pfennig & Murphy 2000, 2002). Additionally, *S. multiplicata* from sympatric and allopatric populations also exhibit canalized differences in their propensity to become carnivores (Pfennig & Murphy 2000, 2002). Our results support Pfennig & Murphy's (2002) conclusion that both facultative and canalized character displacement may be operating in this system.

If plasticity alone were the primary mechanism of character displacement in this system, we might have expected a perfect correspondence between competitive environment and trophic morphology. However, we found that the trophic morphology exhibited by one allopatric population (BP) was very similar to the morphology exhibited by the majority of the sympatric populations, while the trophic morphology of one sympatric population (JA) was more similar to the morphology exhibited by the allopatric populations (Fig. 4.3). Moreover, although the Mantel test indicated a significant correlation between differences in competitive environment and variation in trophic morphology, the strength of the correlation was much less than 1 ($r = 0.43$; see Results). We cannot rule out the possibility that this imperfect correspondence of trophic morphology to competitive environment is due, at least in part, to pond-specific resource

availability. However, our conclusion that facultative character displacement is not the only mechanism at work is reinforced by evidence supporting a role for canalized character displacement in this system. Some closely related sympatric populations share very similar morphology (Fig. 4.4), suggesting that genetic differences underlying the production of the omnivore morph were spread in sympatry via gene flow. Thus, for systems that exhibit exaggerated trait divergence between species in sympatry, it is important to consider that either facultative or canalized character displacement may be operating, potentially even simultaneously. Intraspecific comparative analyses can therefore provide useful information about the evolution of character displacement and its potential consequences.

Table 4.1. Pond locations and sample sizes.

Pond Name	Competitive Environment Type	Latitude (°N)	Longitude (°W)	Cyt *b* sample size	Microsatellite loci sample size
AP	Sympatry	31.680	109.140	17	10
BP	Allopatry	31.885	109.098	20	10
F8	Allopatry	31.933	109.086	10	10
FT	Sympatry	31.740	109.100	11	10
HC	Sympatry	31.734	109.100	13	10
JA	Sympatry	31.818	109.019	13	8
JC	Allopatry	31.929	109.130	20	10
P1	Allopatry	31.901	109.079	19	10
P2	Allopatry	31.914	109.083	9	9
PC	Allopatry	31.670	109.230	14	10
RT	Allopatry	31.939	109.117	20	10
SC	Sympatry	31.691	109.113	10	10
SD	Sympatry	31.813	109.052	8	8
SH	Sympatry	31.768	109.079	19	10
ST	Allopatry	31.910	109.132	15	10
TR	Allopatry	31.930	109.120	28	10
YW	Allopatry	31.645	109.085	20	10

Pond abbreviations: AP, Apache; BP, Bull Pond; F8, Figure Eight; FT, Four Ten; HC, Horned Cow; JA, Javelina; JC, John Carron; PC, Price Canyon; P1, Peach Orchard 1; P2, Peach Orchard 2; RT, Rock Tank; SH, Shrimp; SC, Skeleton Canyon; ST, Starview; SD, Sulfur Draw; TR, Troller; YW, Yucca Wash.

Table 4.2. Trophic morphology by pond.

Pond Name	N	Competitive Environment Type	Mean Morphological Index Score
AP	28	Sympatry	-0.7516
BP	20	Allopatry	-0.8398
F8	18	Allopatry	0.4426
FT	21	Sympatry	-1.2180
HC	13	Sympatry	-0.8157
JA	11	Sympatry	1.3221
JC	20	Allopatry	-0.1389
P1	20	Allopatry	0.1035
P2	24	Allopatry	0.8129
PC	38	Allopatry	0.7449
RT	13	Allopatry	0.4181
SC	35	Sympatry	-0.5033
SD	26	Sympatry	-0.9083
SH	18	Sympatry	-1.3515
ST	20	Allopatry	0.6670
TR	86	Allopatry	0.5160
YW	20	Allopatry	-0.2913

Pond abbreviations: AP, Apache; BP, Bull Pond; F8, Figure Eight; FT, Four Ten; HC, Horned Cow; JA, Javelina; JC, John Carron; PC, Price Canyon; P1, Peach Orchard 1; P2, Peach Orchard 2; RT, Rock Tank; SH, Shrimp; SC, Skeleton Canyon; ST, Starview; SD, Sulfur Draw; TR, Troller; YW, Yucca Wash.

Figure Legends

Figure 4.1. Pond locations in the San Simon Valley of southeastern Arizona and southwestern New Mexico. White circles represent allopatric ponds, and gray circles represent sympatric ponds. Pond name abbreviations and geographical coordinates can be found in Table 4.1.

Figure 4.2. Example neighbor-joining tree topologies for the two extreme scenarios of the evolution of character displacement. S, sympatric population; A, allopatric population. Tadpole drawings represent the expected average *S. multiplicata* tadpole morphology for each population type. a) Example tree topology for scenario under which the omnivore phenotype evolved one time, and then spread to adjacent populations in sympatry. Note that closely related populations share similar tadpole morphology. b) Example tree topology for scenario under which multiple sympatric populations evolve the omnivore phenotype independently. Note that closely related populations do not necessarily share the same tadpole morphology.

Figure 4.3. Dendrogram depicting hierarchical clustering relationships for tadpole trophic morphology among *S. multiplicata* populations in the San Simon Valley. The dendrogram was built using Ward's minimum variance clustering method. The color of the box to the left of the population label indicates that population's morphological index score (see Table 4.2). The morphological index is a multivariate shape variable that includes standardized orbitohyoideus muscle width, standardized interhyoideus muscle width, and degree of mouthpart serration. Populations located nearer to each other on the

dendrogram, with more similar color codes, exhibit similar tadpole trophic morphology. Population abbreviations correspond to Table 4.1. Boldfaced populations are allopatric, and regular typeface populations are sympatric. Note that most allopatric populations (bold) are similar to each other in tadpole trophic morphology (more carnivore-like), and most sympatric populations are similar to each other in tadpole trophic morphology (more omnivore-like).

Figure 4.4. Unrooted neighbor-joining *S. multiplicata* population tree based on Nei's D_A distances calculated from eight microsatellite loci. Proportions next to the nodes indicate bootstrap support (proportion of 2500 bootstrap replicates that included that partition). Population abbreviations correspond to Table 4.1. Similar to Figure 4.3, boldfaced populations are allopatric while regular typeface populations are sympatric. Colored boxes to the left of each population label correspond to each population's morphological index score, as in Figure 4.3.

Figure 4.1

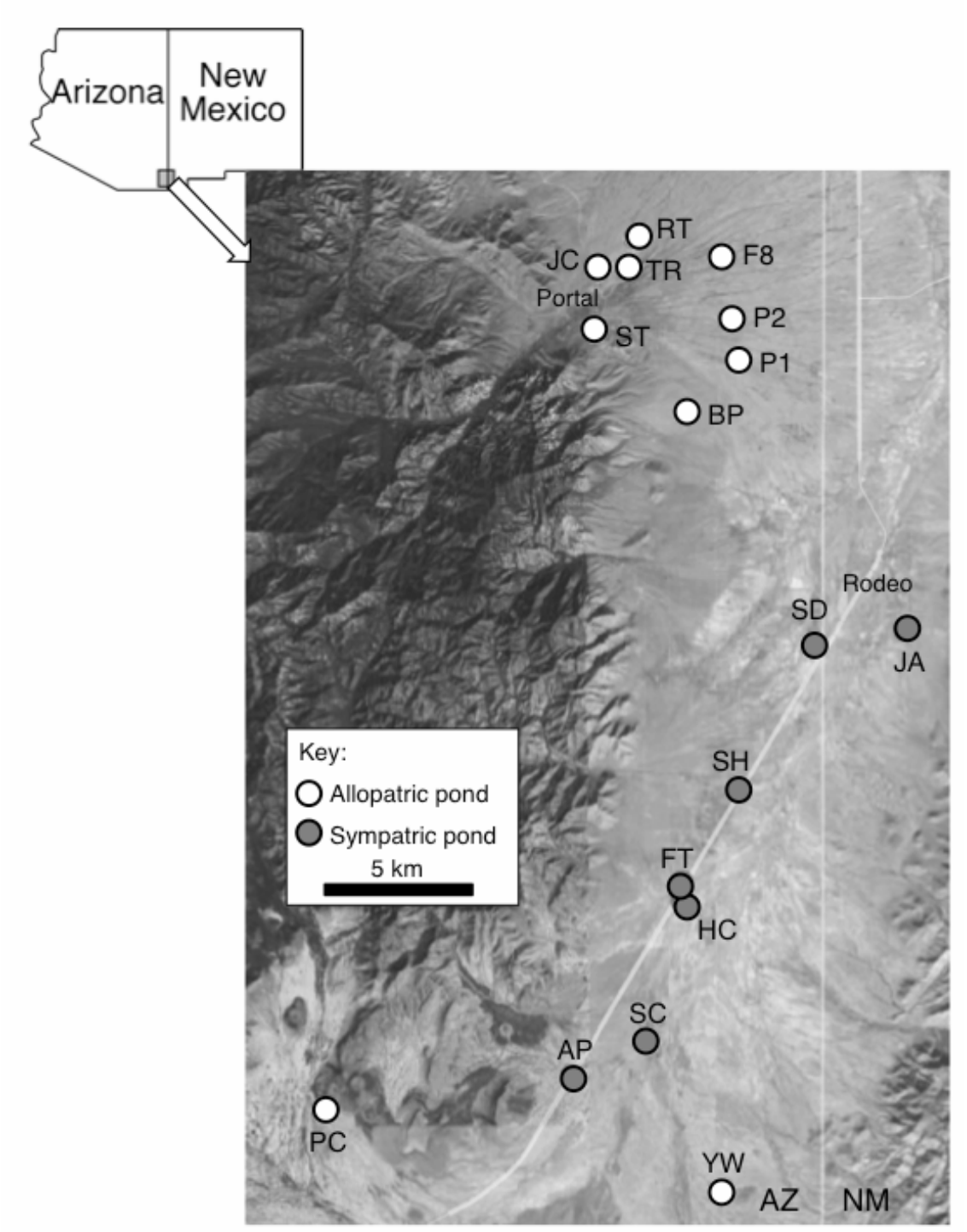
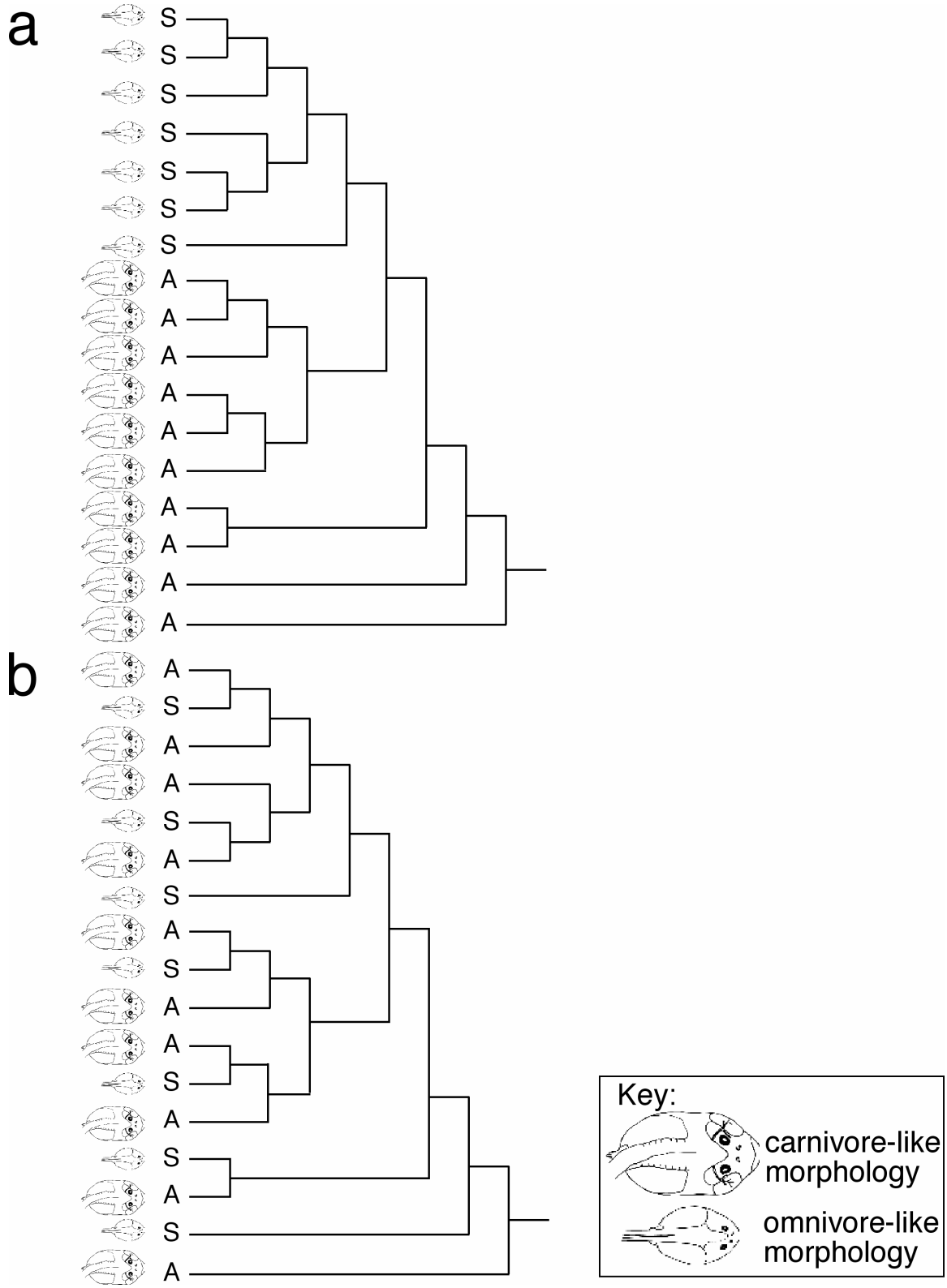

Arizona
New Mexico
RT
JC
TR
F8
Portal
ST
P2
P1
BP
Rodeo
SD
JA
Key:
Allopatric pond
Sympatric pond
5 km
SH
FT
HC
SC
AP
PC
YW
AZ
NM

Figure 4.2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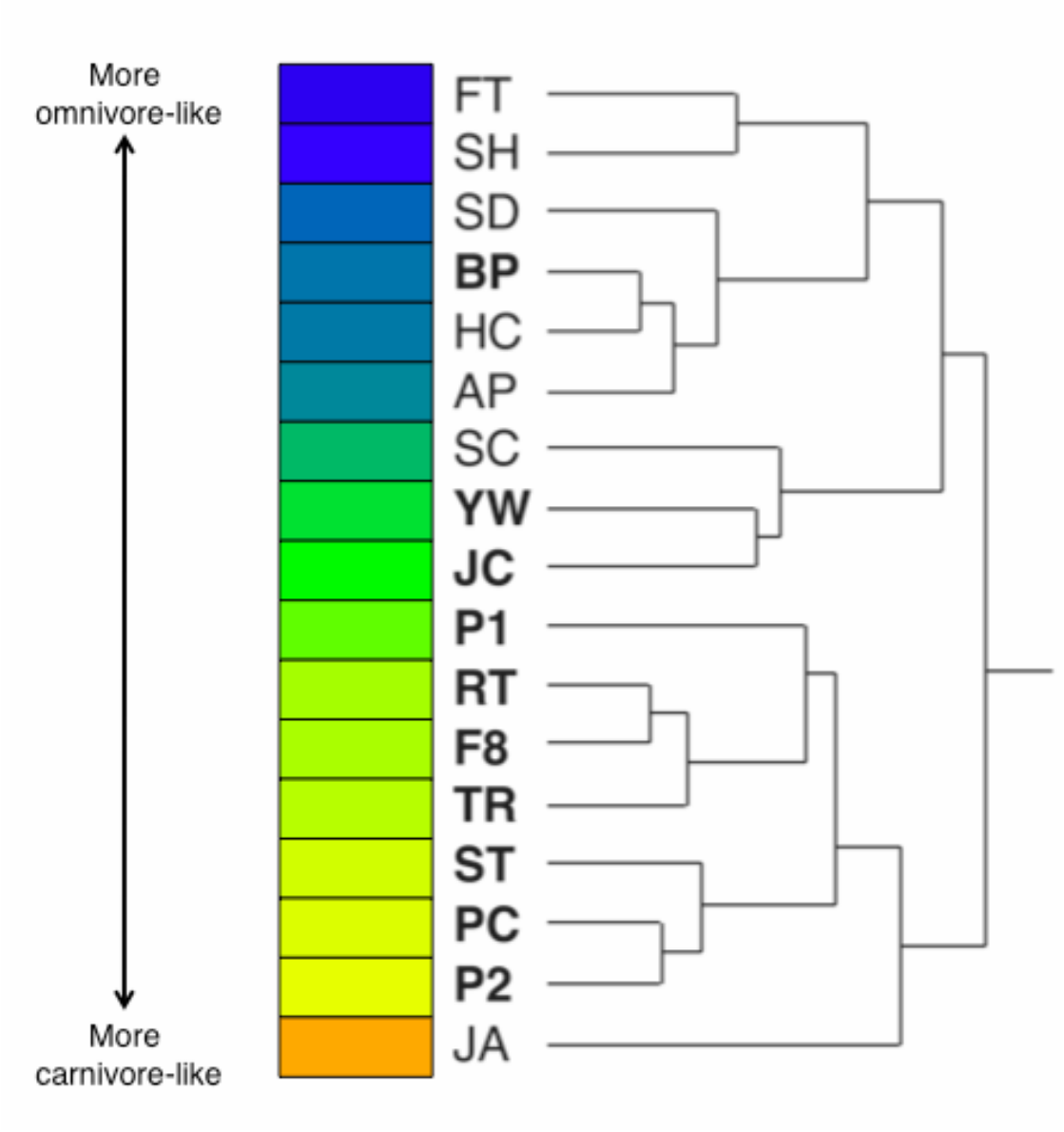
Key:
carnivore-like morphology
omnivore-like morphology

Figure 4.3


More
omnivore-like
FT
SH
SD
BP
HC
AP
SC
YW
JC
P1
RT
F8
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P2
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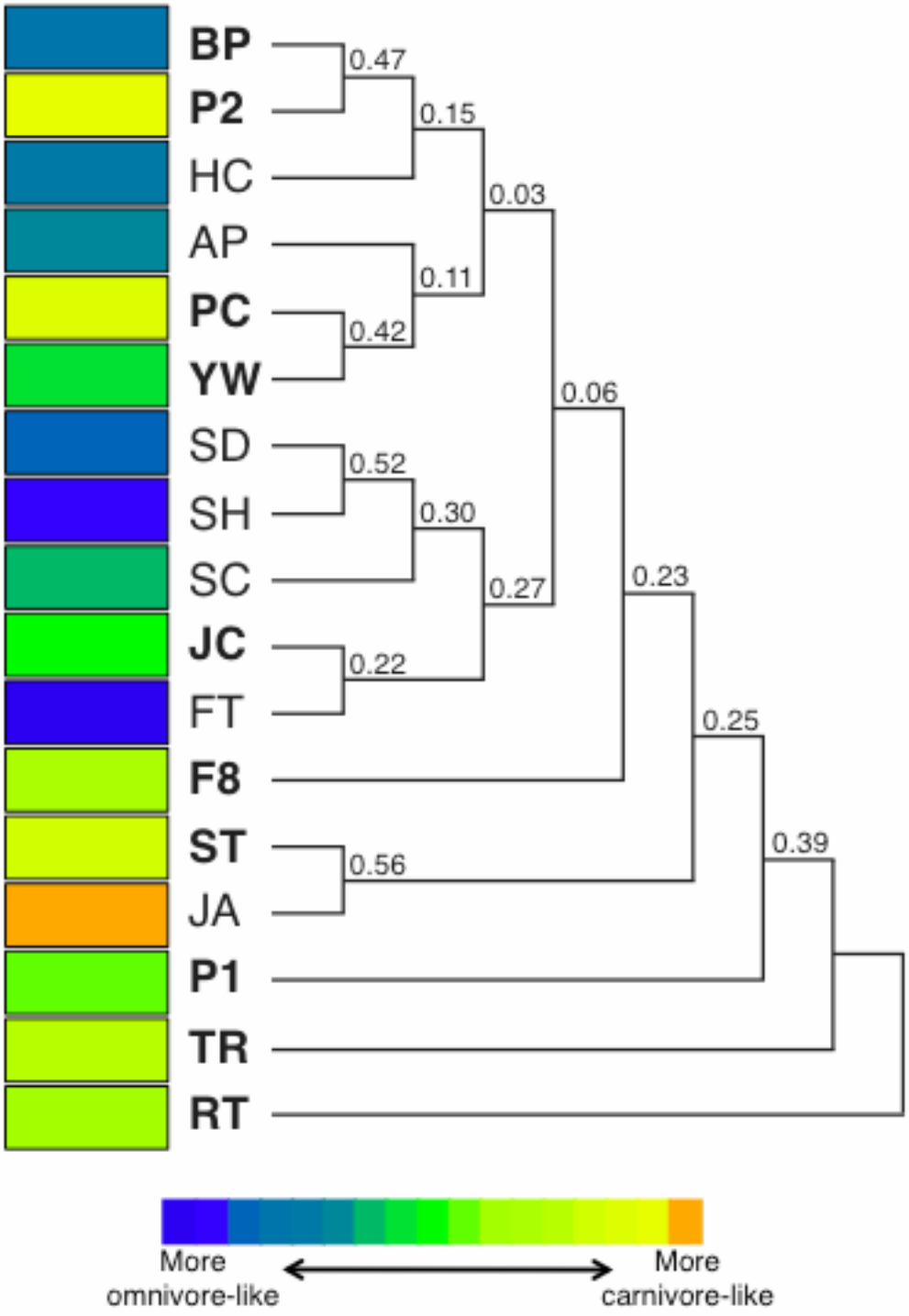
More
carnivore-like

Figure 4.4


BP
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0.47
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0.03
0.11
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0.22
0.25
0.56
0.39
More omnivore-like
More carnivore-like

CHAPTER V

CHARACTER DISPLACEMENT'S ROLE IN SPECIATION: REDUCED GENE FLOW BETWEEN POPULATIONS IN CONTRASTING COMPETITIVE ENVIRONMENTS

Summary

Character displacement – trait evolution stemming from selection to lessen resource competition or reproductive interference between species – has long been regarded as important in finalizing speciation. By contrast, its role in initiating speciation has received less attention. Yet, an indirect consequence of character displacement is that populations in sympatry with the heterospecific experience a different selective environment than those in allopatry. Such divergent selection may favor reduced gene flow between conspecific populations that have undergone character displacement and those that have not, possibly triggering speciation. Here, we explore these ideas by focusing on spadefoot toads, *Spea multiplicata*, which have undergone character displacement, and for which character displacement appears to cause postmating isolation between populations that are in sympatry with a heterospecific and those that are in allopatry. Using mitochondrial sequences and nuclear microsatellite genotypes, we specifically asked whether gene flow is reduced between populations in different selective environments relative to that between populations in the same selective environment. We found a slight, but statistically significant, reduction in gene flow between populations in different selective environments compared to that between

populations in the same environment. These data therefore suggest that speciation may indeed arise as an indirect consequence of character displacement.

Introduction

Closely related species often appear recognizably different where they occur together than where they occur alone (Brown & Wilson 1956; Howard 1993; Schluter 2000b; Dayan & Simberloff 2005). One explanation for this pattern is the process of character displacement (Brown & Wilson 1956), or trait evolution stemming from selection to avoid resource competition or reproductive interference between species. During ecological character displacement, selection favors individuals of each species that are less like the other species in resource use and associated phenotypic traits (reviewed in Schluter 2000b). Ecological character displacement has been documented in numerous taxa (e.g., Fenchel & Kofoed 1976; Losos 1990; Robinson & Wilson 1994; Adams & Rohlf 2000; Pfennig & Murphy 2000; Grant & Grant 2006). Similarly, during reproductive character displacement, selection to lessen interspecific interactions during mating (i.e., hybridization or signal interference) favors individuals of each species with mating characters (e.g., male sexual traits or female preferences) that are less like the other species (reviewed in Coyne & Orr 2004). Reproductive character displacement has also been documented in numerous taxa (e.g., Sætre *et al.* 1997; Rundle & Schluter 1998; Pfennig 2000; Kawano 2002; Higgie & Blows 2007; Smith & Rausher 2008).

Character displacement has long been regarded as playing a central role in species divergence, coexistence, and adaptive radiation (reviewed in Schluter 2000b). Moreover, character displacement has also been acknowledged as an important mechanism for

finalizing speciation (Schluter 2000b; Coyne & Orr 2004). Less frequently considered, however, is character displacement's role in initiating speciation (Hoskin *et al.* 2005; Pfennig & Ryan 2006; Pfennig & Rice 2007; Rice & Pfennig 2007). Yet, an indirect consequence of character displacement is that populations in sympatry with the heterospecific experience a different selective environment than those in allopatry. Local adaptation to these divergent environments may lead to the evolution of reproductive isolation ('ecological speciation'; reviewed in Rundle & Nosil 2005) under either ecological or reproductive character displacement. In the case of ecological character displacement, offspring produced by matings between individuals from different competitive environments may not be well-adapted to competing in either parental environment ('ecologically-dependent postmating isolation'; Rice & Hostert 1993; Hatfield & Schluter 1999; Rundle & Whitlock 2001; Pfennig & Rice 2007). In the case of reproductive character displacement, character displacement may lead to either premating or postmating reproductive isolation. If reproductive character displacement has led to divergence in female preferences or male mating signals between sympatric and allopatric populations, individuals from different environments may not choose, or even recognize, each other as mates (Hoskin *et al.* 2005, Pfennig & Ryan 2006). If individuals from different environments do mate, however, any male offspring produced may exhibit intermediate sexual signals, and therefore be less successful at obtaining mates (e.g., Höbel & Gerhardt 2003; reviewed in Servedio & Noor 2003), while any female may exhibit preferences that are inappropriate for her environment (reviewed in Servedio & Noor 2003). For both ecological and reproductive character displacement, then, selection

should favor individuals that avoid mating between environments, leading to a predicted reduction in gene flow between conspecific populations in sympatry and allopatry.

Here, we explore these ideas empirically by focusing on spadefoot toads, *Spea multiplicata*. As we describe in detail below, this species has undergone both ecological and reproductive character displacement in areas where it co-occurs with a heterospecific, *S. bombifrons*. As we also describe below, previous work suggests that character displacement has resulted in postmating isolation between populations of *S. multiplicata* that are in sympatry with *S. bombifrons* and those that are in allopatry. We specifically used this system to test whether gene flow is reduced between conspecific populations in different competitive environments relative to that between populations in the same competitive environment.

Study System

Mexican spadefoot toads, *Spea multiplicata*, and Plains spadefoot toads, *S. bombifrons*, co-occur in the San Simon Valley of southeastern Arizona and southwestern New Mexico (Fig. 5.1). In this region, both species co-occur below 1350 m in elevation (hereafter termed "sympatry"), while at higher elevations, only *S. multiplicata* is present (hereafter termed "allopatry"; Pfennig *et al.* 2006). *Spea* spend much of the year in underground burrows, emerging during the summer rainy season to feed and to breed (Bragg 1944, 1945). Spadefoots breed on the evening following a rainstorm, in ephemeral ponds formed by run-off (Bragg 1945). Females choose a mate based on male call characteristics, which signify potential fitness benefits for her offspring (Pfennig

2000). Female spadefoots breed at most once per year, while males may attend several breeding aggregations if multiple rainstorms occur (Tinsley 1989).

Larvae of both species exhibit trophic polyphenism. They may develop into either a small-headed omnivore morph, which feeds mostly on organic detritus on the pond bottom, or a large-headed, morphologically distinct carnivore morph, which specializes on anostracan fairy shrimp in the water column (Pomeroy 1981; Pfennig 1990, 1992). The carnivore morph is induced by the ingestion of shrimp (Pomeroy 1981; Pfennig 1990).

These two species exhibit ecological character displacement in tadpole morph production (Pfennig & Murphy 2000, 2003; Pfennig *et al.* 2006, 2007). In ponds where each species occurs alone, both species produce similar, intermediate frequencies of each morph. However, in sympatric ponds, *S. multiplicata* produce almost entirely omnivores, whereas *S. bombifrons* produce almost entirely carnivores (Pfennig & Murphy 2000, 2003; Pfennig *et al.* 2006). Experiments reveal that this sympatric divergence in morph production has evolved because of interspecific resource competition (Pfennig & Murphy 2002; Pfennig *et al.* 2007). Moreover, this divergence is due, at least in part, to canalized differences in morph production between sympatric and allopatric populations (Pfennig & Murphy 2002).

In southeastern Arizona, individual *S. multiplicata* tadpoles in allopatric versus sympatric ponds experience divergent competitive conditions (absence vs. presence of *S. bombifrons*) across distances of only 5-30 km (Fig. 5.1). These different conditions have resulted in divergent patterns of selection for populations of *S. multiplicata* in the two competitive environments (Pfennig *et al.* 2007). Specifically, in allopatry, intraspecific

competition leads to disruptive selection favoring extreme omnivore and carnivore morphs. In contrast, sympatric *S. multiplicata* tadpoles experience stabilizing selection favoring intermediate trophic morphology (Pfennig *et al.* 2007). Moreover, offspring produced by matings between individuals from allopatry and sympatry (hereafter "between environment offspring" or BE offspring) have reduced fitness relative to offspring produced by matings within the same competitive environment (hereafter "within environment offspring" or WE offspring; Pfennig & Rice 2007). A controlled experiment indicates that much of this postmating isolation stems from ecological selection against BE offspring. Resulting in part from their intermediate trophic morphology, BE offspring were competitively inferior in both allopatric and sympatric competitive environments (Pfennig & Rice 2007). Thus, as a by-product of ecological character displacement, populations of *S. multiplicata* in sympatry and allopatry with *S. bombifrons* may be evolving reproductive isolation.

In addition to ecological character displacement, *Spea multiplicata* and *S. bombifrons* have undergone reproductive character displacement in both male call rates and female preferences. In sympatry, male call rates for the two species have diverged relative to male call rates in allopatry (Pierce 1976; K. Pfennig unpubl. data). Moreover, allopatric female *S. multiplicata* prefer males with a fast call rate; however, sympatric female *S. multiplicata* prefer males with an average call rate (Pfennig 2000). Faster *S. multiplicata* calls resemble *S. bombifrons* calls; therefore, female *S. multiplicata* that choose average males avoid costly hybridization (Simovich *et al.* 1991; Pfennig & Simovich 2002). Likewise, sympatric female *S. bombifrons* discriminate against *S. multiplicata* males

under conditions in which hybridization is costly, whereas allopatric females do not (Pfennig 2007).

To summarize, in southeastern Arizona and southwestern New Mexico, the spadefoot toad species *S. multiplicata* and *S. bombifrons* exhibit both ecological and reproductive character displacement (Pierce 1976; Pfennig 2000; Pfennig & Murphy 2000, 2003; Pfennig *et al.* 2006, 2007; Pfennig 2007). Moreover, experimental evidence suggests that ecological character displacement has resulted in postmating reproductive isolation between *S. multiplicata* populations in sympatry and in allopatry (Pfennig & Rice 2007). We therefore predicted that gene flow should be reduced between sympatric and allopatric *S. multiplicata* populations, relative to gene flow between ponds within each environment. In order for postmating reproductive isolation to lead to reduced gene flow, however, there must be an opportunity for selection to act on BE offspring (Nosil *et al.* 2003). Thus, there must be some migration between competitive environments. Therefore, we first assessed general levels of gene flow in *S. multiplicata*. After finding evidence supporting the occurrence of migration (see Results), we used a population genetic approach to test our prediction that gene flow between populations in sympatry and allopatry should be reduced.

Methods

To determine whether gene flow is reduced between ponds in different competitive environments relative to gene flow between ponds within the same competitive environments, we asked three questions. First, is there a sufficient level of overall gene flow in the spadefoot toad system to allow postmating reproductive isolation to operate?

Second, is the population structure consistent with reduced gene flow between sympatry and allopatry? Third, when controlling for geographic distance, is there a correlation between competitive environment and either population structure or estimates of gene flow?

Sampling

We collected *S. multiplicata* tadpoles during summers 1999-2004 in southeastern AZ and southwestern NM. Tadpoles were sampled seven to 15 days posthatching from random sites throughout natural, temporary ponds using a hand-held dip net. We sampled ten allopatric ponds and eight sympatric ponds (Fig. 5.1; Table 5.1). Within a few hours of collection, tadpoles were killed by immersion in a 0.1% aqueous solution of tricane methanesulfonate (MS 222) and preserved in 95% ethanol. For each pond site, we used Google Earth version 4.2.0198.2451 (beta) to determine latitudinal and longitudinal coordinates. The geographic coordinates were used to calculate geographic distance between each pair of pond sites using the great circle formula as implemented by the GPS WAYPOINT REGISTER'S distance calculator (http://www.gpswaypoints.co.za/Downloads/distcalc.xls).

DNA Extraction, Amplification, Sequencing, and Genotyping

We used two procedures for extracting DNA. For tadpoles collected from 1999-2001, we extracted genomic DNA using the DNeasy Blood & Tissue Kit (QIAGEN), following the manufacturer's protocol for extractions from animal tissue samples. For tadpoles collected from 2002-2004, we incubated tissues overnight with Proteinase K (QIAGEN),

extracted DNA using a saturated NaCl solution, and precipitated and washed the DNA using ethanol.

We amplified and sequenced a portion of the cytochrome *b* (cyt *b*) gene from the mitochondrial genome (mtDNA). We used a forward primer designed from an *S. multiplicata* sequence (SCB1-F; 5'- TCCCAACCCCATCTAACATC-3') and a reverse primer designed from a *Xenopus laevis* sequence (XCB2-R; 5'-GAGGGCTAAGATTAGGATGGATA-3'). We carried out 40 cycles of the polymerase chain reaction on the MJ Research PTC-200 DNA Engine thermal cycler using the following profile: 94 ºC for 30 s; 50 ºC for 30 s; 72 ºC for 90 s. The amplification products were purified using ExoSAP-IT® (USB). After purification, we submitted the amplification products to the UNC-Chapel Hill Genome Analysis Facility for direct sequencing on an ABI 3730 Genetic Analyzer. We obtained cyt *b* sequences from a total of 275 individuals (8-28 individuals per pond; Table 5.1). Using SEQUENCHER 4.5 (GeneCodes), we assembled the sequence chromatograms for each sample into contigs and proofread the sequences. We then aligned all the sequences using CLUSTALX 1.83 (Thompson *et al.* 1997), and trimmed them to a length of 663 base pairs using MACCLADE 4.08 (Maddison & Maddison 1989). We used both PAUP* 4.0b10 (Swofford 2002) and MACCLADE 4.08 to group the sequences into unique haplotypes (These cyt *b* sequences were previously published in Rice & Pfennig 2008; GenBank accession nos. EU285643-EU285652, EU285654, EU285657; Table A.4).

For eight to ten individuals per pond (Table 5.2), we amplified eight previously published microsatellite loci (three di-nucleotide loci: *Sm1*, *Sm4*, *Sm23*; two tri-nucleotide loci: *Sb15*, *Sb28*; three tetra-nucleotide loci: *Sm14*, *Sm20*, *Sm25*; GenBank

Accession Numbers EU285444-EU285445, EU285450-EU285452, EU285454-EU285456) using the published protocols (Rice *et al.* in press). We submitted the amplified products to the UNC-Chapel Hill Genome Analysis Facility for genotyping on an ABI 3730 Genetic Analyzer. Peaks were scored based on an internal size standard (GeneScan™-500 LIZ®; Applied Biosystems) using GeneMapper v3.7 (Applied Biosystems).

Genetic variation, Hardy-Weinberg equilibrium, null alleles, and linkage disequilibrium

For cyt *b*, we used ARLEQUIN 3.11 (Schneider *et al.* 2000) to calculate haplotype diversity and nucleotide diversity for each pond.

For the microsatellite loci, we used MICROSATELLITE ANALYZER (MSA) 4.05 (Dieringer & Schlötterer 2003) to calculate number of alleles, observed (H_O) and expected heterozygosities (H_E), and allelic richness (standardized allele number by pond sample size to allow comparisons across ponds; Ar) for each pond. Using the probability test in GENEPOP 4.0.6 (Rousset 2008), we tested each locus for Hardy-Weinberg Equilibrium (HWE). Statistical significance was estimated using the Markov chain method, with 10,000 dememorizations, 1000 batches, and 10,000 iterations per batch. Because tests for HWE were performed for each pond-locus combination, we adjusted the a-value for each locus using sequential Bonferroni corrections (Rice 1989). Because a number of pond-locus combinations were not in HWE (see Results), we used MICRO-CHECKER (van Oosterhout *et al.* 2004) to test for the possible presence of null alleles, one possible cause for departure from HWE. Significance was estimated using 1000 randomizations and Bonferroni-corrected significance levels. Two loci (*Sm4* and *Sb15*)

exhibited signatures of a null allele (see Results). Null alleles may affect the accuracy of estimates of population structure and gene flow (Selkoe & Toonen 2006). Two ways that null alleles may be dealt with include excluding them from analyses or statistically correcting for their presence. Because one of the loci that exhibited signatures of a null allele was also one of our most variable loci (*Sm4*, see Table 5.2), and therefore valuable for detecting subtle population structure, we used the Oosterhout correction algorithm in MICRO-CHECKER to generate corrected genotype and allele frequencies for use where possible. We also tested for linkage disequilibrium between all pairs of loci across all ponds using Fisher's global test in GENEPOP 4.0.6 (Rousset 2008).

Estimating overall gene flow

In order to assess opportunity for postmating reproductive isolation, we used population structure as an indicator of gene flow. We used ARLEQUIN 3.11 (Schneider *et al.* 2000) to calculate global F_{ST} (or Φ_{ST} for the sequence data) using an Analysis of Molecular Variance (AMOVA). Each pond was treated as a separate population. We calculated F_{ST} / Φ_{ST} separately for the microsatellite data and the cyt *b* sequences. For cyt *b*, we used the Tamura (1992) model of DNA substitution as the method of calculating distances between haplotypes. The Tamura (1992) model was the closest of the methods used by Arlequin to the HKY model, which was previously identified as the most likely model for our cyt *b* data (Rice & Pfennig 2008). Significance was estimated using 10,000 permutations of the data.

We used one additional estimator of overall gene flow for the microsatellite data. When F_{ST} is calculated from highly variable markers, such as microsatellites, the

maximum possible value is often much less than one (Nagylaki 1998; Hedrick 1999). Thus, microsatellite data will produce F-statistics that suggest lower population structure, and higher gene flow, than actual values (Nagylaki 1998; Hedrick 1999). Therefore, for the microsatellite data, we used GENEPOP 4.0.6 (Rousset 2008) to calculate overall gene flow (Nm) using Slatkin's (1985) private allele method, which performs better than F_{ST} when variation is high (Hedrick 1999).

Testing for reduced gene flow between competitive environments

Hierarchical population structure

If gene flow is reduced between populations in contrasting environments, then we predicted that populations within each environment should be more similar to each other in genotype frequencies than they are to populations in the opposite environment. To test this prediction, we calculated hierarchical F-statistics with an Analysis of Molecular Variance (AMOVA) in ARLEQUIN 3.11 (Schneider *et al.* 2000), once using only the microsatellite data set, and once using the cyt *b* sequence data. F-statistics indicate the proportion of the overall total variance in genotype frequencies that is partitioned within populations (F_{ST} / Φ_{ST}) and within defined groups of populations (F_{CT} / Φ_{CT}). We defined two population groups: allopatry and sympatry (Fig. 5.1, Table 5.2). Therefore, the F_{CT} / Φ_{CT} values indicate whether gene flow is reduced between competitive environments relative to the gene flow within each environment. For the microsatellite data, we also used a locus-by-locus AMOVA to estimate F_{ST} and F_{CT} for each locus individually. We also calculated F_{ST} and F_{CT} for all eight microsatellite loci separately a second time, using the allele frequency data type in Arlequin, which allowed us to correct *Sm4* and *Sb15* for

the presence of null alleles. We were then able to directly compare population structure estimates calculated with and without correcting for null alleles. Significance of the F-statistics (against the null hypothesis of zero) was estimated using 50,000 permutations of the data.

Correlations between gene flow and competitive environment

If gene flow is reduced between populations in contrasting competitive environments, we predicted that population structure should tend to be higher, and gene flow lower, between ponds in different environments versus between ponds in the same environment. To test this prediction, we used partial Mantel tests (Smouse *et al.* 1986) in ARLEQUIN 3.11. This method tests for partial correlations among distance matrices by creating a null distribution of correlation coefficients from permutations of the data. We performed separate partial Mantel tests for each of several indicators of gene flow: 1) pairwise F_{ST} values based on all eight microsatellite loci; 2) pairwise coalescent-based maximum likelihood gene flow estimates, based on all eight microsatellite loci; and 3) pairwise coalescent-based Bayesian gene flow estimates based on cyt *b* and all eight microsatellite loci. Details of the analyses that generated these values are below. The first predictor matrix included log-transformed geographic distances (km) between ponds. The second predictor matrix was categorical, and coded for the environment comparison between ponds (i.e., 0=both ponds are either allopatric or sympatric, 1=one pond is allopatric and one is sympatric). To estimate significance, we used 100,000 permutations.

To calculate the pairwise F_{ST} values, we used MSA 4.05 (Dieringer & Schlötterer 2003). We chose to include all eight loci because the presence of null alleles at *Sm4* and *Sb15* did not appear to greatly affect population structure estimates (see Results).

We calculated coalescent-based maximum likelihood gene flow estimates between each pond pair using MIGRATE 2.3 (Beerli & Felsenstein 1999; Beerli & Felsenstein 2001). MIGRATE implements a Markov Chain Monte Carlo (MCMC) simulation to perform a Maximum Likelihood search. We included all eight microsatellite loci. Because of input file requirements, however, we were unable to use the corrected genotype frequencies for *Sm4* and *Sb15*. Each MIGRATE run estimated the gene flow between one pair of ponds. We used the stepwise mutation model, which is the standard model used for microsatellite data in MIGRATE. After a burn-in of 10,000 trees per chain, we sampled 100,000 trees, of which 5000 were recorded, for each of 15 short chains. The short chains were followed by 4 long chains, for which 50,000 trees were recorded from 2,000,000 sampled. We averaged over the long chains, and allowed the program to automatically increase chain length until the genealogy acceptance rate reached 10%. Each analysis estimated theta ($4N_e\mu$, where N_e is effective population size and μ is mutation rate) and M (m/μ, where m is the migration rate) in each direction between two populations. Therefore, to obtain estimates of gene flow (Nm), we multiplied theta by M and divided by 4. Because MIGRATE estimates migration between populations in both directions, we averaged the two Nm values between each pond pair for the partial Mantel analysis.

We used LAMARC 2.1.2b (Kuhner 2006; Kuhner & Smith 2007) to estimate gene flow using a Bayesian analysis of the combined microsatellite and cyt *b* data. We used the F84

model for the cyt *b* data, and the Brownian-motion model for the microsatellite data. Again because of input file requirements, we were unable to correct *Sm4* and *Sb15* for null alleles. For this analysis, we grouped the ponds sites into 11 pond groups (6 in allopatry, 5 in sympatry; Table 5.3). We grouped neighboring ponds (within 3.5 km) that had pairwise F_{ST} values that were both less than 0.05 and not significantly different after Bonferroni corrections. Latitude and longitude coordinates for each population group were determined by averaging the coordinates of each of the ponds in the group (Table 5.3). Geographic distances between groups were calculated as before (see above). Unlike the pairwise MIGRATE estimates, we used LAMARC to estimate gene flow between all the group pairs in one single analysis. For each locus, our search included two replicates with the following search strategy: one initial chain, with 500 samples and a sampling interval of 20 steps, and one final chain, with 10,000 samples and a sampling interval of 20 steps. We discarded 1000 samples for burn-in. Similar to MIGRATE, LAMARC estimates both theta and M in each direction between two populations; therefore, to obtain estimates of gene flow (Nm), we multiplied M by theta for the recipient population and divided by 4. For the partial Mantel analysis, we averaged the two Nm values between each pond pair.

Results

Genetic variation, Hardy-Weinberg equilibrium, null alleles, and linkage disequilibrium

Pond-specific genetic variation measures for the cyt *b* locus and the eight microsatellite loci are listed in Tables 5.1 and 5.2, respectively. Most pond-loci combinations did not significantly depart from HWE (Table 5.4); however, *Sm4* departed from HWE in six of the 18 ponds, while *Sb15* showed departure from HWE in 13 of the

18 ponds. Similarly, when we tested for the presence of null alleles, these two loci exhibited significant signatures of a null allele in at least 50% of the ponds (Table 5.4). None of the loci were in linkage disequilibrium ($P > 0.37$ for all loci pairs across all ponds).

Estimating overall gene flow

Based on the cyt *b* sequence data, global $\Phi_{ST} = 0.156$ ($P < 0.0001$), while for the microsatellite loci, the global $F_{ST} = 0.043$ ($P < 0.0001$).

Based on the microsatellite data only, Slatkin's private allele method estimated the average effective number of migrants exchanged between local populations (Nm) at 3.79, after correcting for sample size. The mean frequency of private alleles (p(1)) was 0.0603. This Nm estimate is lower than the Nm value corresponding to the global F_{ST} calculated from the microsatellite data (Nm = 5.6) using the equation $F_{ST} = 1/(1+4Nm)$, suggesting that high variability in the microsatellite loci has resulted in an underestimation of population structure. In general, these data suggest that *S. multiplicata* are dispersing enough to provide opportunity for postmating reproductive isolation to act.

Testing for reduced gene flow between contrasting competitive environments

Hierarchical population structure

Table 5.5 presents the results of the locus-by-locus AMOVA estimates of F_{ST} and F_{CT}. For all eight microsatellite loci combined, significant population structure was evident ($F_{ST} = 0.045$, $P < 0.00001$; Table 5.5), suggesting that gene flow among the ponds in the San Simon Valley is lower than would be expected under panmixia. This

significant population structure was evident across all loci except *Sb15* (Table 5.5), whether analyzed as microsatellite genotypes or as allele frequencies. Significant population structure was also evident in the cyt *b* sequence data ($\Phi_{ST} = 0.149$, $P < 0.00001$). These data therefore suggest that these populations show consistent differentiation across both mitochondrial and nuclear genomes.

In the microsatellite data, we found evidence that a slight, but significant, proportion of the variance in genotypes can be explained by the allopatric and sympatric pond groupings ($F_{CT} = 0.005$, $P = 0.0296$; Table 5.5), suggesting that there may be a slight reduction in gene flow between ponds in contrasting competitive environments. This pattern appears to be driven by differentiation at the loci *Sm4*, *Sm14*, *Sm20*, and *Sm23*, although the F_{CT}s for *Sm14* and *Sm20* loci are marginally non-significant (Table 5.5). In contrast, these groupings do not explain any of the variance in the cyt *b* sequences ($\Phi_{CT} = -0.016$, $P = 0.81$).

Qualitative comparisons of F_{ST} and F_{CT} calculated from the uncorrected *Sm4* and *Sb15* microsatellite genotypes and from the corrected allele frequencies indicate that the null alleles did not have large effects on our population structure estimates (Table 5.5), especially relative to the differences seen in the other loci in which no correction was made. In all cases except one, the statistical significance of the estimates remained similar. The one exception was for the *Sm4* F_{CT} estimate, which changed from being significantly different from zero to marginally non-significantly different from zero (Table 5.5). When the uncorrected microsatellite genotype frequencies were analyzed, the *Sm4* $F_{CT} = 0.015$ ($P = 0.0399$); however, when the allele frequencies were corrected for the null allele, $F_{CT} = 0.012$ ($P = 0.0844$). In sum, there is a suggestion of slightly reduced

gene flow between ponds in contrasting competitive environments; however, it is unclear how much of this pattern results from a null allele at *Sm4*.

Correlations between gene flow and competitive environment

We found a marginally non-significant positive correlation between type of competitive comparison and pairwise F_{ST}, which was stronger after we controlled for the effect of geographic distance between ponds (without controlling for distance: r = 0.06, *P* = 0.11; controlling for distance: r = 0.11, *P* = 0.07). This is suggestive that there may be a slight reduction in gene flow between populations in sympatry and allopatry. However, there was no evidence of a correlation between type of competitive comparison and Nm, when estimated from either the MIGRATE analysis of the microsatellite data (without controlling for distance: r = -0.08, *P* = 0.88; controlling for distance: r = -0.06, *P* = 0.79) or the LAMARC analysis of both data sets combined (without controlling for distance: r = 0.11, *P* = 0.10; controlling for distance: r = 0.08, *P* = 0.25).

Discussion

In the spadefoot toad system, which has undergone both ecological and reproductive character displacement, we asked two questions in order to evaluate whether character displacement may be indirectly leading to the evolution of reproductive isolation between populations of *Spea multiplicata* in sympatry and allopatry with *S. bombifrons*. First, is there enough gene flow among pond sites to provide an opportunity for selection to act against BE offspring? A controlled lab experiment detected both ecologically-dependent and intrinsic postmating isolation between *S. multiplicata* populations in sympatry and

allopatry (Pfennig & Rice 2007). However, because spadefoot toads live in a desert environment, and only emerge from their burrows a couple of nights a year during the summer rainy season (Bragg 1944, 1945), their opportunities for dispersal may be extremely limited. Second, given a sufficient level of gene flow to suggest that postmating isolation may have the opportunity to act, we asked whether levels of gene flow between ponds in different environments were reduced relative to gene flow between ponds within the same environment. In the spadefoot toad system, divergent patterns of selection are likely between sympatric and allopatric populations because of both ecological and reproductive character displacement (Pierce 1976; Pfennig 2000; Pfennig & Murphy 2000, 2003; Pfennig *et al.* 2006, 2007; Pfennig 2007). Such conditions may lead to reproductive isolation, and therefore to reduced gene flow.

Two indicators of overall gene flow suggest that postmating isolation should have an opportunity to act in this system. Although we found evidence of significant global population structure based on both the mitochondrial (cyt *b* $\Phi_{ST} = 0.156$, $P < 0.0001$) and nuclear data (microsatellite $F_{ST} = 0.043$, $P < 0.0001$), the levels of population structure present suggested that average gene flow among the pond sites is fairly high (around five migrants per generation). The gene flow estimate from Slatkin's (1985) private allele method was lower, at 3.8 migrants per generation, but still indicated a significant amount of gene flow. These results indicate that matings between individuals from sympatry and allopatry should be occurring frequently enough to allow selection to operate against BE offspring.

It may be argued that these levels of gene flow are so high that they will overwhelm selection against matings between competitive environments, thereby preventing the

evolution of reproductive isolation (Servedio & Kirkpatrick 1997). There are at least two reasons why the evolution of reproductive isolation between *S. multiplicata* populations in sympatry and allopatry remains possible, however. First, the levels of gene flow among ponds are likely lower than the Nm estimates derived from F_{ST} or Φ_{ST} indicate. Accuracy of migration estimates derived from F-statistics depends on assumptions that are not generally met in natural populations (Whitlock & McCauley 1999). Coalescent-based approaches such as those implemented in MIGRATE (Beerli & Felsenstein 1999) and LAMARC (Kuhner 2006) rely on fewer assumptions. In our study, the median value for Nm calculated by MIGRATE based only on the microsatellite markers was 1.33, while the median value for Nm calculated by LAMARC based on both microsatellite and mitochondrial markers was 0.52. Both of these estimates were much lower than the Nm estimates derived from F_{ST}. Even though using F_{ST} to estimate number of migrants is problematic, F_{ST} may still be useful for providing both an overall picture of the effects of gene flow and estimates of relative gene flow (Neigel 2002), which is how we employed F_{ST} in this study.

The second reason why the evolution of reproductive isolation between sympatric and allopatric *S. multiplicata* populations remains possible is that selection against mating with an individual from the opposite environment should be strong. Pfennig & Rice (2007) calculated that the relative reduction in fitness exhibited by the BE tadpoles should project to a 16% reduction in fecundity for adult females. The observed reduction in growth rate (Pfennig & Rice 2007) may also impact tadpole survival (Pfennig & Pfennig 2005). Moreover, because of the reproductive character displacement in this system, any adult *S. multiplicata* with one parent from sympatry and one parent from

allopatry may either make poor mate choices (in the case of females) or have low mating success because of an intermediate call rate (in the case of males). Therefore, in this system, conditions should be favorable for the evolution of reproductive isolation (Servedio & Kirkpatrick 1997): There should be enough gene flow to allow selection to operate against offspring produced by matings between environments, but not so much that it will overwhelm the strength of divergent selection between environments (Nosil *et al.* 2003).

The population structure estimate from the mtDNA was much higher than the estimate from the nuclear microsatellite data. One explanation for this disparity between genomes is that males may disperse more than females. Indeed, discrepancies between population structure estimates based on mitochondrial and nuclear data are often used to infer sex-biased dispersal (e.g., Baker *et al.* 1998; Gibbs *et al.* 2000; Harper & Pfennig 2008; reviewed in Prugnolle & deMeeus 2002). Such results should be interpreted with caution, however (Prugnolle & deMeeus 2002). Another factor that may lead to such a discrepancy is the variation in effective population size for the two types of markers. Theoretically, the effective population size of the mitochondrial genome is four times smaller than that of the nuclear genome. Genetic drift will therefore act more strongly on mitochondrial genomes, which could lead to a greater degree of population structure. On the other hand, when mating is not random, which is often the case in natural populations, the expected relationship between genome effective population sizes does not hold (Chesser & Baker 1996). Another potential explanation for the observed population structure discrepancy is that the F_{ST} estimate from the microsatellite loci may be reduced because of high variation at these loci (Nagylaki 1998; Hedrick 1999). Hedrick (1999)

proposed an equation that calculates the maximum possible value of F_{ST} given the level of variation and number of populations sampled (see equation 2a in Hedrick 1999). Using that equation, for *S. multiplicata*, the maximum possible F_{ST} is 0.310; our value of F_{ST} (F_{ST} = 0.043) is therefore 13.9% of this maximum value, which is very close to the Φ_{ST} calculated for cyt *b* (Φ_{ST} = 0.156). Male-biased dispersal, differences in genome effective population size, and marker variation may all be responsible for the observed discrepancy in population structure between cyt *b* and the microsatellite markers. We cannot rule out these alternative explanations. Because of the spadefoot toad mating system, however, male-biased dispersal may be a likely cause for at least part of the discrepancy in this case: Each summer, males potentially have more opportunities to mate, and therefore disperse, than females (Tinsley 1989).

Two lines of evidence suggest that gene flow is reduced between *S. multiplicata* populations in different selective environments; i.e., populations in sympatry with *S. bombifrons* and those in allopatry. First, for the microsatellite data, we found that sympatric and allopatric pond groupings explained a significant portion of the variance in genotype frequencies (F_{CT} = 0.005, P = 0.0296). This suggests that the ponds within each environment are slightly more similar to each other in genotype frequencies than they are to ponds in the other environment, which is what would be expected if gene flow were reduced between environments. When this F_{CT} value is corrected for highly variable markers (see above; Hedrick 1999), the allopatric and sympatric groupings account for 1.6% of the variance in genotype frequencies. Second, when controlling for differences in geographic distance, we found a marginally non-significant relationship between pond pairwise F_{ST} and the type of environment comparison (r = 0.11, P = 0.07), suggesting that

differentiation between ponds in different selective environments was slightly higher (and gene flow lower) than between ponds in the same selective environment. This relationship was non-significant, so it must be interpreted with care. However, it is consistent with the results from the hierarchical population structure analysis.

In contrast to the microsatellite (nuclear) data, the cyt *b* (mitochondrial) data did not show a similar signature of reduced gene flow between sympatric and allopatric populations. This discrepancy between the results from the two genomes may be explained in at least two ways. First, if male-biased dispersal is occurring in this system (see above), females may not be under selection to reduce their dispersal further. Therefore, we would not expect to see a reduction of gene flow in the mitochondrial genome, which is passed only from mother to offspring. Second, the mutation rate for cyt *b* is likely much lower than for the microsatellite loci. Because of this, cyt *b* provides a picture of gene flow in the more distant past relative to the microsatellite markers. If the postmating isolation results from recent contact between *S. multiplicata* and *S. bombifrons* (A. Chunco, unpubl. data), the microsatellite data would be more likely to detect any resulting effects on gene flow.

Given multiple reasons why selection should favor reproductive isolation between populations of *S. multiplicata* in sympatry with *S. bombifrons* and those in allopatry (see Study System), why did we not detect a larger reduction in gene flow? Oscillating selection is one possible explanation. BE offspring may perform better in some years versus others, if, for instance, resources are more plentiful (e.g., Gibbs & Grant 1987). Even if selection against BE offspring were very strong in most years, bouts of relaxed selection would lessen the average effect detectable through population genetic data.

Similarly, pulsed migration would also dampen any signature of reduced gene flow. During very heavy rains in the San Simon Valley, adult spadefoot toads may get swept away in running water (A. Rice, personal observation), and moved to the opposite environment. Finally, if selection against BE offspring is recent, the analyses we used may not be able to detect any resulting reduction in gene flow. Data from museum records suggest that the contact between *S. multiplicata* and *S. bombifrons* in the San Simon Valley may be recent (A. Chunco, unpubl. data). Therefore, selection may not have had time to produce a large reduction in gene flow. Methods for detecting contemporary gene flow, such as STRUCTURE (Pritchard *et al.* 2000) and BAYESASS+ (Wilson & Rannala 2003), should be better able to detect any recent gene flow patterns.

Alternatively, we may not have detected a large reduction in gene flow between *S. multiplicata* populations in sympatry and allopatry relative to gene flow within these environments because such a reduction may not be present. Instead, selection against BE offspring may select for an overall reduction in migration. Yukilevich and True (2006) found that when ecologically-dependent postmating isolation is present, migration modification should be an important mechanism of speciation. Moreover, in general, systems undergoing character displacement may already be under selection for decreased overall gene flow so that adaptation to the sympatric environment is not swamped out by migration from allopatry, and vice versa (Pfennig & Pfennig 2005). In such systems, even if selection favored it, an additional reduction in gene flow between sympatric and allopatric environments may not evolve any faster than the overall reduction of gene flow favored as a consequence of local adaptation.

In conclusion, we found evidence that character displacement may indirectly promote the evolution of reproductive isolation and reduced gene flow between conspecific populations in sympatry and allopatry. However, in systems undergoing character displacement, a further reduction in gene flow between these two environments may prove to be less prevalent than overall reductions in migration. Future work will be necessary to determine the general mechanisms by which character displacement ignites speciation.

Table 5.1. Pond geographic locations and cytochrome *b* sample sizes and variation summaries.

Pond	Latitude (°N)	Longitude (°W)	N	No. Haplotypes	Haplotype diversity (S.D.)	Nucleotide diversity (S.D.)
AP	31.680	109.140	17	3	0.2279 (0.1295)	0.000892 (0.000840)
BP	31.885	109.098	20	2	0.3368 (0.1098)	0.000509 (0.000589)
F8	31.933	109.086	10	2	0.2000 (0.1541)	0.001516 (0.001254)
FT	31.740	109.100	11	2	0.1818 (0.1436)	0.001658 (0.001323)
HC	31.734	109.100	13	4	0.6026 (0.1306)	0.001279 (0.001087)
JA	31.818	109.019	13	2	0.3846 (0.1321)	0.002335 (0.001675)
JC	31.929	109.130	20	2	0.3947 (0.1006)	0.000596 (0.000648)
P1	31.901	109.079	19	2	0.1988 (0.1121)	0.000300 (0.000436)
P2	31.914	109.083	9	3	0.5556 (0.1653)	0.000923 (0.000908)
PC	31.670	109.230	14	3	0.2747 (0.1484)	0.000432 (0.000547)
PO	31.766	109.077	9	4	0.5833 (0.1833)	0.001007 (0.000961)
RT	31.939	109.117	20	6	0.7789 (0.0646)	0.004500 (0.002740)
SC	31.691	109.113	10	1	0	0
SD	31.813	109.052	8	3	0.4643 (0.2000)	0.002278 (0.001737)
SH	31.768	109.079	19	2	0.4561 (0.0852)	0.000689 (0.000709)
ST	31.910	109.132	15	2	0.4762 (0.0920)	0.000719 (0.000739)
TR	31.930	109.120	28	8	0.8280 (0.0448)	0.003877 (0.002386)
YW	31.645	109.085	20	2	0.1895 (0.1081)	0.000286 (0.000423)

Pond abbreviations: AP, Apache; BP, Bull Pond; F8, Figure Eight; FT, Four Ten; HC, Horned Cow; JA, Javelina; JC, John Carron; PC, Price Canyon; PO, Post Office Canyon; P1, Peach Orchard 1; P2, Peach Orchard 2; RT, Rock Tank; SH, Shrimp; SC, Skeleton Canyon; ST, Starview; SD, Sulfur Draw; TR, Troller; YW, Yucca Wash.

Table 5.2. Summary of genetic variation at each microsatellite locus for each pond site.

Pond	Environ-ment type	N	Measure of variation	*Sm1*	*Sm4*	*Sm14*	*Sm20*	*Sm23*	*Sm25*	*Sb15*	*Sb28*
AP	S	10	N_A	3	4	6	6	5	7	5	3
			Ar	3.00	3.99	5.57	5.93	4.60	6.34	4.57	2.97
			Range	105-125	167-201	190-242	163-191	151-161	143-191	50-77	314-320
			H_O/H_E	0.60/0.65	0.40/0.74	1.00/0.81	0.60/0.82	0.70/0.75	0.70/0.79	0.70/0.68	0.60/0.59
BP	A	10	N_A	4	7	4	5	5	6	3	4
			Ar	3.96	6.53	3.80	4.60	4.60	5.40	3.00	3.40
			Range	103-125	165-191	198-242	163-199	151-163	143-171	50-56	281-326
			H_O/H_E	0.60/0.72	0.80/0.81	0.90/0.68	0.60/0.66	0.50/0.75	0.80/0.74	0.40/0.69	0.30/0.28
F8	A	10	N_A	4	4	5	4	8	5	4	2
			Ar	3.77	3.80	4.60	3.80	7.33	4.80	3.96	2.00
			Range	103-125	165-191	178-234	179-195	135-161	155-187	50-65	314-320
			H_O/H_E	0.60/0.59	0.60/0.67	0.90/0.73	0.70/0.70	1.00/0.86	1.00/0.79	0.30/0.66	0.90/0.52
FT	S	10	N_A	5	6	6	7	7	5	6	4
			Ar	4.20	5.60	5.76	6.56	6.49	4.56	5.57	3.80
			Range	105-127	165-191	162-238	171-195	135-165	159-175	50-92	314-347
			H_O/H_E	0.30/0.37	0.50/0.82	0.60/0.84	0.80/0.86	0.50/0.67	0.60/0.57	0.60/0.81	0.60/0.61
HC	S	10	N_A	4	8	6	6	8	4	4	4
			Ar	3.80	6.96	5.94	5.76	7.50	3.53	3.97	3.60
			Range	103-125	159-191	186-242	163-195	149-167	159-191	50-59	314-326
			H_O/H_E	0.60/0.70	0.30/0.74	0.86/0.80	0.80/0.82	0.80/0.88	0.30/0.28	0.70/0.72	0.70/0.54
JA	S	8	N_A	5	7	8	7	7	7	6	5
			Ar	5	7	8	7	7	7	6	5
			Range	103-125	159-191	186-242	163-195	131-167	143-183	44-68	314-332
			H_O/H_E	0.50/0.53	0.38/0.78	1.0/0.85	0.86/0.90	0.75/0.69	0.50/0.82	0.38/0.81	0.50/0.61
JC	A	10	N_A	2	8	7	6	8	6	6	4
			Ar	1.97	7.17	6.16	5.74	7.53	5.76	5.40	3.74
			Range	105-125	163-201	170-238	171-199	133-167	143-191	50-65	314-326

			H_O/H_E	0.20/0.19	0.50/0.86	0.60/0.69	1.00/0.83	0.80/0.89	0.40/0.84	0.60/0.78	0.50/0.44
P1	A	10	N_A	4	8	5	6	7	8	4	2
			Ar	3.80	7.53	4.60	5.60	6.37	7.11	4.00	2.00
			Range	103-125	165-201	186-238	163-195	137-167	143-191	50-65	314-320
			H_O/H_E	0.40/0.64	0.50/0.89	0.40/0.73	0.90/0.81	0.60/0.82	0.60/0.75	0.50/0.76	0.30/0.48
P2	A	9	N_A	5	5	6	7	9	5	7	4
			Ar	4.77	4.89	5.67	6.77	8.32	4.78	6.65	3.99
			Range	103-125	165-191	186-238	163-195	133-163	143-171	50-68	314-326
			H_O/H_E	0.67/0.61	0.33/0.76	0.78/0.73	0.89/0.88	0.78/0.80	0.89/0.67	0.56/0.78	0.67/0.73
PC	A	10	N_A	4	7	9	6	6	6	6	4
			Ar	3.60	6.70	8.31	5.40	5.57	5.70	5.93	3.79
			Range	103-125	165-191	186-238	171-199	149-161	143-175	50-68	314-332
			H_O/H_E	0.90/0.60	0.30/0.87	0.70/0.78	0.60/0.76	0.60/0.80	0.50/0.73	0.30/0.85	0.70/0.56
PO	S	9	N_A	4	7	7	5	8	6	4	5
			Ar	3.88	7.00	6.65	4.89	7.71	5.76	4.00	4.67
			Range	103-125	163-201	158-242	175-195	131-167	143-175	50-65	311-326
			H_O/H_E	0.50/0.61	0.50/0.69	0.67/0.81	0.78/0.80	0.75/0.86	0.56/0.73	0.44/0.78	0.78/0.67
RT	A	10	N_A	3	8	7	8	10	7	4	4
			Ar	2.80	7.67	6.73	7.49	9.07	6.20	4.00	3.60
			Range	105-125	165-201	186-242	163-195	133-167	155-179	50-65	314-341
			H_O/H_E	0.50/0.42	0.50/0.90	1.00/0.87	0.78/0.86	0.80/0.91	0.70/0.76	0.30/0.76	0.40/0.54
SC	S	10	N_A	3	6	6	6	7	5	5	3
			Ar	2.80	5.70	5.56	5.60	6.16	4.74	4.74	2.80
			Range	105-125	165-201	186-238	171-195	131-159	143-171	47-65	314-326
			H_O/H_E	0.20/0.36	0.50/0.73	0.80/0.78	0.80/0.82	0.70/0.69	0.70/0.70	0.40/0.72	0.10/0.35
SD	S	8	N_A	4	5	6	5	3	4	5	4
			Ar	4.00	5.00	6.00	5.00	3.00	4.00	5.00	4.00
			Range	103-125	165-191	186-238	163-195	149-153	155-175	50-65	314-329
			H_O/H_E	0.50/0.53	0.50/0.80	0.75/0.80	0.88/0.73	0.38/0.69	0.50/0.70	0.50/0.79	0.63/0.52
SH	S	10	N_A	3	6	7	5	6	5	6	3
			Ar	2.94	5.37	6.67	4.94	5.70	4.76	5.56	2.77
			Range	103-125	165-201	178-238	163-195	131-155	159-191	47-65	314-320

			H_O/H_E	0.40/0.36	0.40/0.71	0.80/0.84	0.80/0.79	1.00/0.73	0.90/0.74	0.60/0.76	0.30/0.28
ST	A	10	N_A	4	6	6	7	10	7	6	4
			Ar	3.77	5.93	5.40	6.44	8.96	6.19	5.73	3.57
			Range	103-125	167-191	190-242	171-195	133-167	151-191	50-65	314-344
			H_O/H_E	0.70/0.55	0.60/0.85	0.60/0.76	0.70/0.74	1.00/0.92	0.60/0.73	0.40/0.81	0.40/0.36
TR	A	10	N_A	5	7	7	8	8	6	7	4
			Ar	4.57	6.59	6.20	7.16	7.36	5.20	6.39	3.97
			Range	103-125	165-189	186-242	163-195	137-165	155-179	47-65	314-326
			H_O/H_E	0.80/0.65	0.30/0.87	0.90/0.78	1.00/0.85	0.80/0.87	0.80/0.62	0.80/0.84	0.80/0.74
YW	A	10	N_A	5	10	6	5	5	3	5	5
			Ar	4.57	8.93	5.76	4.80	4.96	2.99	4.96	4.56
			Range	103-129	163-199	174-242	175-191	151-161	155-171	50-65	314-329
			H_O/H_E	0.50/0.62	0.70/0.91	0.80/0.84	0.70/0.81	0.40/0.76	0.40/0.49	0.30/0.82	0.60/0.57
Locus Totals			N_A	10	18	16	10	15	13	11	12
			Ar	3.98	7.73	6.75	6.31	7.34	5.84	5.38	3.86
			Range	103-129	159-201	158-242	163-199	131-167	143-191	44-92	281-347

Environment type: S, Sympatric Ponds; A, Allopatric Ponds. Measures of variation: N_A, Number of Alleles; Ar, Allelic richness; H_O, Observed heterozygosity; H_E, Expected heterozygosity.

Table 5.3. Pond groupings for the LAMARC analysis.

Ponds in group	**Environment type**	**Latitude (°N)**	**Longitude (°W)**
BP	A	31.885	109.098
JC, ST	A	31.920	109.131
F8, P1, P2	A	31.916	109.083
PC	A	31.670	109.230
RT, TR	A	31.934	109.118
YW	A	31.645	109.085
AP, SC	S	31.685	109.127
FT, HC	S	31.737	109.100
JA, SD	S	31.815	109.035
PO	S	31.767	109.077
SH	S	31.768	109.079

Environment type: S, Sympatric Ponds; A, Allopatric Ponds.

Table 5.4. Results of tests for departure from Hardy-Weinberg Equilibrium and the presence of null alleles.

Pond	Environment type	*Sm1*	*Sm4*	*Sm14*	*Sm20*	*Sm23*	*Sm25*	*Sb15*	*Sb28*
AP	S	n.s.	**0.0013**†	n.s.	n.s.	n.s.	n.s.	**0.0039**	n.s.
BP	A	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**0.0077**†	n.s.
F8	A	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**0.0016**†	n.s.
FT	S	n.s.	n.s. †	n.s.	n.s.	n.s.	n.s.	**<0.0001**	n.s.
HC	S	n.s.	**<0.0001**†	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
JA	S	n.s.	**0.0022** †	n.s.	n.s.	n.s.	n.s.	**0.0001**†	n.s.
JC	A	n.s.	**0.0001**†	n.s.	n.s.	n.s.	**<0.0001**†	**0.0011**	n.s.
PC	A	n.s.	**<0.0001**†	n.s.	n.s.	n.s.	n.s.	**<0.0001**†	n.s.
PO	S	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s. †	n.s.
P1	A	n.s.	n.s. †	n.s. †	n.s.	n.s.	n.s.	**0.0014**	n.s.
P2	A	n.s.	n.s. †	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
RT	A	n.s.	n.s. †	n.s.	n.s.	n.s.	n.s.	**0.0001**†	n.s.
SH	S	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
SC	S	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**0.0039**†	n.s. †
ST	A	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**0.0005**†	n.s.
SD	S	n.s.	n.s.	n.s.	n.s.	n.s. †	n.s.	**0.0072**	n.s.
TR	A	n.s.	**<0.0001**†	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
YW	A	n.s.	n.s.	n.s.	n.s.	n.s. †	n.s.	**0.0006**†	n.s.
No. Ponds Showing Significant Departure from HWE		0	6	0	0	0	1	13	0
No. Ponds With Signature of Null Allele		0	10	1	0	2	1	9	1

†: Significant signature of a null allele, based on 1000 randomizations and Bonferroni corrected significance levels in MICRO-CHECKER
Environment type: S, Sympatric Ponds; A, Allopatric Ponds.

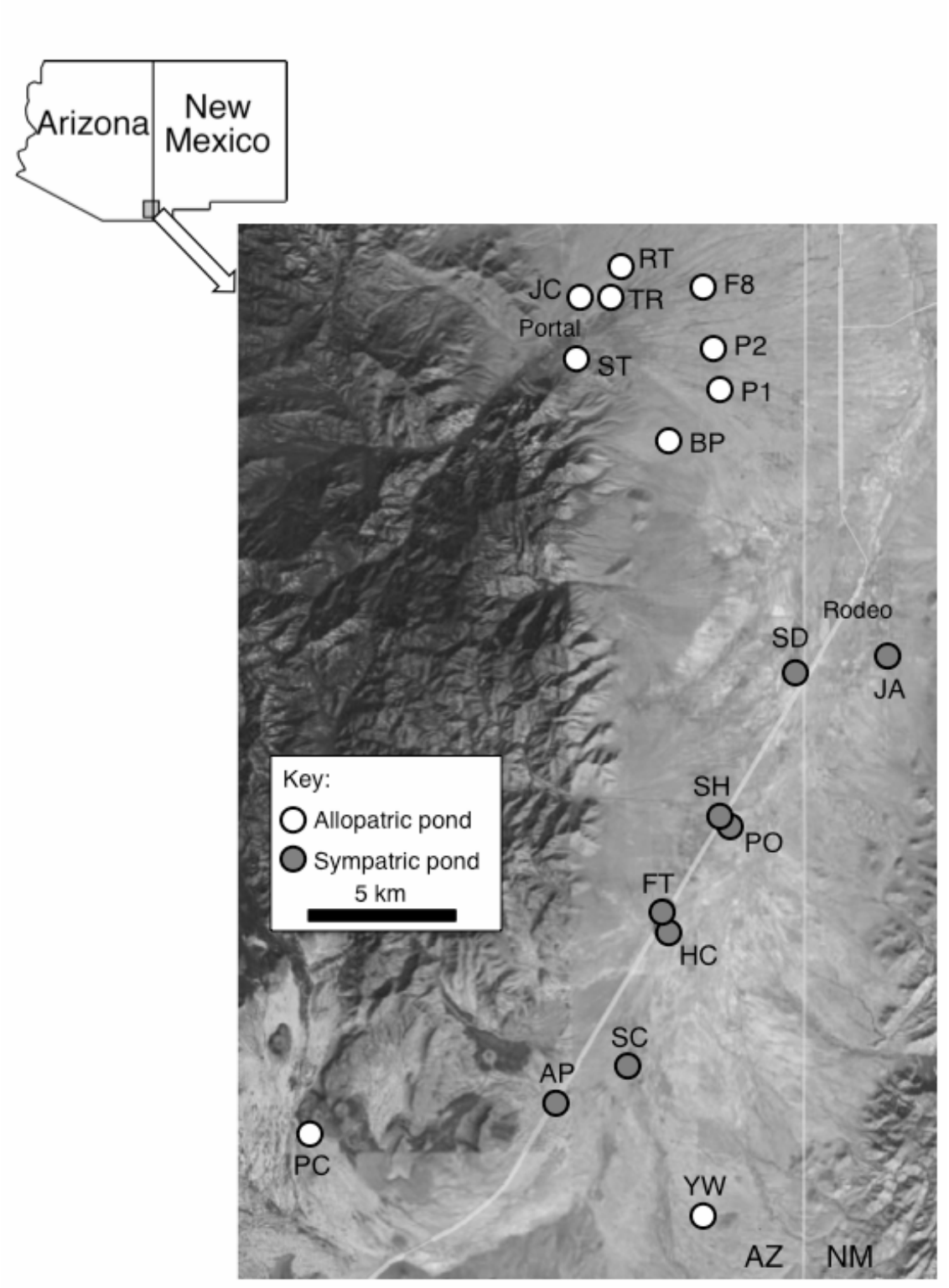
Table 5.5. Hierarchical population structure results.

	Sm1	*Sm4*	*Sm14*	*Sm20*	*Sm23*	*Sm25*	*Sb15*	*Sb28*	Combined
F_{ST}- Uncorrected Microsatellite Data (*P*-value)	**0.046 (0.0004)**	**0.087 (<0.0001)**	**0.028 (0.0005)**	**0.067 (<0.0001)**	**0.037 (0.0003)**	**0.041 (0.0004)**	0.014 (0.6451)	**0.030 (0.0041)**	**0.045 (<0.0001)**
F_{ST}- Corrected Allele Frequency Data (*P*-value)	**0.043 (0.0007)**	**0.075 (<0.0001)**	**0.028 (0.0029)**	**0.073 (<0.0001)**	**0.037 (0.0004)**	**0.041 (<0.0001)**	-0.008 (0.7198)	**0.030 (0.0080)**	
F_{CT}- Uncorrected Microsatellite Data (*P*-value)	-0.006 (0.7049)	**0.015 (0.0399)**	0.006 (0.0932)	0.009 (0.1080)	**0.016 (0.0089)**	-0.002 (0.5587)	-0.002 (0.5801)	-0.008 (0.8934)	**0.005 (0.0296)**
F_{CT}- Corrected Allele Frequency Data (*P*-value)	-0.007 (0.7452)	0.012 (0.0844)	0.005 (0.1124)	0.010 (0.1010)	**0.016 (0.0116)**	-0.002 (0.5560)	-0.003 (0.7859)	-0.008 (0.8914)	

Figure Legend

Figure 5.1. Pond locations in the San Simon Valley of southeastern Arizona and southwestern New Mexico. White circles represent allopatric ponds, and gray circles represent sympatric ponds. Pond name abbreviations and geographical coordinates can be found in Table 5.1.

Figure 5.1


Arizona
New Mexico
RT
JC
TR
F8
Portal
ST
P2
P1
BP
Rodeo
SD
JA
Key:
Allopatric pond
Sympatric pond
5 km
SH
PO
FT
HC
SC
AP
PC
YW
AZ
NM

CHAPTER VI

CONCLUSIONS

Ecological character displacement has long been acknowledged as an important mechanism driving adaptive radiation and species coexistence. Indeed, this process has been well studied in many taxa. Yet, many questions concerning the causes and consequences of character displacement remain unanswered. The research I have presented in this thesis addresses several of these questions. At the same time, it suggests avenues of future research that may further illuminate the evolution of ecological character displacement and its consequences.

First, by distinguishing between two non-exclusive routes to character displacement— ISE and sorting (chapter II) —we can gain a better understanding of: 1) why character displacement may be more likely to occur in some species than others; 2) why character displacement may proceed more quickly in some cases than others; and 3) why some cases of character displacement may ignite speciation while others do not. Answers to these questions will provide insight into the relative likelihood of the two possible outcomes of competition— coexistence and competitive exclusion.

In chapter II, I described several promising avenues of research that will allow a distinction to be made between ISE and sorting in systems undergoing character displacement. One of the more exciting suggestions is to make geographic comparisons of levels of neutral variation linked to genomic regions that are associated with adaptation to the competitor species. Levels of linked variation can provide information

about whether or not a selective sweep has recently occurred at those genomic regions. If ISE has been more important, there should be no signature of a selective sweep in allopatric populations, while there should be a strong signature of a sweep in sympatric populations. Alternatively, if sorting has been important, there should be evidence of sweeps in both sympatric and allopatric populations. Although such an approach currently is not feasible for many systems that may be undergoing character displacement, a recent review notes that a growing number of studies in wild populations have begun to address the genetic basis of traits underlying adaptation (Ellegren & Sheldon 2008). Indeed, such research is already being pursued in a species that is well known for undergoing character displacement— the threespine stickleback *Gasterosteus aculeatus* (Albert *et al.* 2007).

When resources are asymmetric in quality, character displacement may lead to differential fitness consequences between competing species, creating a "winner" and a "loser." Indeed, although the species that is displaced to the lower quality resource (the loser) benefits by avoiding competitive exclusion, the fitness costs of this displacement may increase the risk of Darwinian extinction for this species in sympatry. Such a case of differential fitness consequences has occurred for *Spea bombifrons* (the winner) and *S. multiplicata* (the loser) in southeastern Arizona (Pfennig & Pfennig 2005). In chapter III, I found that the winner (*S. bombifrons*) is a more recent invader into the region where character displacement occurred. Moreover, historical selection in its ancestral range may have pre-adapted *S. bombifrons* for monopolizing the superior resource.

I suggested that when resources are asymmetric, the invading species might generally win during character displacement. Successful invaders may often be superior

competitors (Sakai *et al.* 2001; Vila & Weiner 2004; Rehage *et al.* 2005), and therefore may be more likely to win during character displacement. Moreover, we may only detect character displacement when the invader monopolizes the more profitable resource; because population sizes should be smaller for recent invaders in general, any invaders that fail to monopolize the more profitable resource are more likely to go extinct. In most cases of character displacement, we do not know which species is the more recent invader or whether displacement to alternative resources leads to differential fitness consequences. Thus, before we can determine whether order of invasion can predict the outcome of character displacement in general, a great deal of additional research into known cases of character displacement must be performed. Furthermore, invasive species may provide an excellent opportunity to test the prediction that invaders may be more likely to win during character displacement. Each species invasion may provide a natural experiment by which to investigate factors that affect the outcome of character displacement.

Although character displacement has been documented in many taxa, whether or not the divergence between species is replicated across sympatric populations has not been well established. In chapter IV, I used morphological and population genetic data to show that populations of *S. multiplicata* have independently undergone character displacement. However, I also found some evidence of non-independence for a few populations. This research therefore underscored the importance of testing for evolutionary independence in cases of character displacement.

Several methods exist for testing for replicated character displacement, including spatial autocorrelation (Edwards & Kot 1995), Mantel tests (Mantel 1967, Smouse *et al.*

1986), and intraspecific contrasts (Felsenstein 2002). However, besides spadefoot toads, these tests have been performed in few potential cases of character displacement (but see Hansen *et al.* 2000; Marko 2005). In the future, more effort should be made to establish the independence of populations undergoing character displacement. Moreover, the necessary tests are becoming more accessible: A newly available program (Migsel; available at http://evolution.gs.washington.edu/migration/migsel.html) can implement Felsenstein's (2002) model of intraspecific contrasts. In addition to providing more robust evidence for character displacement and the role of competition in divergence, such testing will also be important for understanding whether and why certain populations or species may undergo character displacement more easily than others.

Character displacement has long been regarded as important in finalizing speciation, but its role in initiating speciation has received less attention. In chapter V, I used population genetics to explore whether populations of *S. multiplicata* in sympatry and allopatry may be evolving reproductive isolation. I found evidence of a slight, but statistically significant, reduction in gene flow between populations in different selective environments, which suggests that speciation may arise as an indirect consequence of character displacement.

Given that *S. multiplicata* is undergoing both ecological and reproductive character displacement, it was surprising that the observed reduction in gene flow was not larger. This may be because an overall reduction in migration, not just a reduction between the two selective environments, was favored (Yukilevich & True 2006). Indeed, because character displacement is a form of local adaptation, species undergoing character displacement may already be under selection for low overall gene flow (Pfennig &

Pfennig 2005). I suggested that in such systems, selection against mating between selective environments might not lead to any reduction in gene flow beyond the already low levels. Future research will be necessary to determine whether this is generally the case. One way to test this would be to compare levels of historical gene flow among populations estimated with coalescent-based methods, like LAMARC (Kuhner 2006), to levels of contemporary gene flow estimated with Bayesian assignment methods such as STRUCTURE (Pritchard *et al.* 2000) or BAYESASS+ (Wilson & Rannala 2003). Contemporary gene flow that is lower than historical gene flow would be consistent with selection favoring low dispersal in species undergoing character displacement. On the other hand, if either or both contemporary and historical gene flow estimates showed relatively reduced gene flow between selective environments, this would be consistent with selection against mating between environments leading to reproductive isolation.

My thesis research suggested that: 1) Distinguishing between two routes to character displacement will provide a better understanding of when competition will lead to coexistence over competitive exclusion; 2) Invading species may generally win during character displacement; 3) In order to better understand how character displacement evolves, it is important to test for independent divergence among sympatric populations; and 4) Speciation may arise as an indirect consequence of character displacement. My research has addressed several questions related to the causes and consequences of character displacement. Yet, my results have also stimulated ideas for future research—research that will ultimately provide further insight into the process that Charles Darwin (1859) described so long ago: divergence of character, or character displacement.

APPENDIX[3]

Chapter III Supplemental Methods

DNA Extraction, Amplification, and Sequencing

We used two procedures for extracting DNA. For tadpoles collected from 1999-2001 and 2005-2006, and for all adult tissues, we extracted genomic DNA using the DNeasy Blood & Tissue Kit (QIAGEN), following the manufacturer's protocol for extractions from animal tissue samples. For tadpoles collected from 2002-2004, we incubated tissues overnight with Proteinase K (QIAGEN), extracted DNA using a saturated NaCl solution, and precipitated and washed the DNA using ethanol. We then amplified and sequenced a portion of the cytochrome *b* (cyt *b*) gene from the mitochondrial genome (mtDNA). We used a forward primer designed from an *S. multiplicata* sequence (SCB1-F; 5'-TCCCAACCCCATCTAACATC-3') and a reverse primer designed from a *Xenopus laevis* sequence (XCB2-R; 5'-GAGGGCTAAGATTAGGATGGATA-3'). We carried out 40 cycles of the polymerase chain reaction on the MJ Research PTC-200 DNA Engine thermal cycler using the following profile: 94 ºC for 30 s; 50 ºC for 30 s; 72 ºC for 90 s. The amplification products were purified using ExoSAP-IT® (USB). After purification, we submitted the amplification products to the UNC-Chapel Hill Genome Analysis Facility for direct sequencing on an ABI 3730 Genetic Analyzer. We sequenced an average of 15.5 *S. multiplicata* individuals from each of 27 locations (5-36 individuals

[3] This appendix is modified from the online supplemental material published with Rice, A. M. and Pfennig, D. W. 2008. Analysis of range expansion in two species undergoing character displacement: why might invaders generally 'win' during character displacement? *Journal of Evolutionary Biology* 21: 696-704. The supplementary material is available at http://www.blackwell-synergy.com/doi/abs/10.1111/j.1420-9101.2008.01518.x.

per location; Table A.2), and an average of 6.4 *S. bombifrons* individuals from each of 54 locations (1-33 individuals per location; Table A.1).

Using SEQUENCHER 4.5 (GeneCodes), we assembled the sequence chromatograms for each sample into contigs and proofread the sequences. We then aligned all the sequences using CLUSTALX 1.83 (Thompson *et al.* 1997), and trimmed them to a length of 663 base pairs using MACCLADE 4.08 (Maddison & Maddison 1989). We used both PAUP* 4.0b10 (Swofford 2002) and MACCLADE 4.08 to group the sequences into unique haplotypes (GenBank accession nos. EU285613-EU285657; Tables A.3 and A.4).

Determining Order of Invasion

In order to calculate the relative population sizes of *S. multiplicata* and *S. bombifrons* in the past, we estimated Θ (= $2N_f\mu$, where N_f = effective number of females in the population, and μ = mutation rate per site per generation) and exponential growth rates (g, in units of μ^{-1}) for each of the two species in SE AZ using LAMARC 2.1.2b (Kuhner 2006). We used $\mu = 4.0 \times 10^{-9}$ substitutions per site per generation as our mutation rate (Tan & Wake 1995). We substituted our estimates of Θ, g, and μ into the following equation to solve for Θ 50,000 generations in the past:

$$\Theta_{50,000} = \Theta e^{-g\mu(50,000)}$$

We then divided $\Theta_{50,000}$ by Θ to get the relative N_f at 100,000 years in the past. Values of relative N_f greater than one indicate the population has decreased in size over time, while values of N_f less than one indicate the population has increased in size over time.

We calculated relative N_f using multiple estimates of *g*: the point estimate, and the endpoints of the 95% HPD. We used the point estimate of Θ for all of the calculations.

Chapter III Supplemental Results

Overall Patterns of Diversity

From 344 *S. bombifrons* samples obtained from across the range (Fig. 3.1, Table A.1), we sequenced 663 base pairs of cyt *b*. A total of 34 polymorphic sites yielded 30 unique haplotypes (Fig. 3.3, Table A.3). Most of these haplotypes were unique to a geographic location (67%; Table A.3). Two common haplotypes were present, however. One of these haplotypes (haplotype 1, Table A.3) was found in all geographic regions except the Southwestern region. Indeed, in the Northern region, haplotype 1 was the only haplotype we sampled. The other (haplotype 3, Table A.3) was found only in the Southwestern region; yet, it was found in all 16 ponds sampled within that region.

From 419 *S. multiplicata* samples collected from SE AZ and SW NM (Fig. 3.1, Table A.2), we found a total of 30 polymorphic sites (out of 663 bp), yielding 15 unique haplotypes (Fig. 3.3). We found one common and widespread haplotype (haplotype 32, Table A.4), present in 26 of the 27 ponds we sampled. Of the 15 haplotypes we sampled, 7 were found at only one geographic site (47%; Table A.4).

Table A.1. *Spea bombifrons* collection locations and sample sizes. The ten ponds marked as syntopic were included in the IBD analysis reported in the paper. Population group assignments were used in determining *S. bombifrons'* ancestral range, and are abbreviated as follows: N, Northern group; C, Central group; SW, southwestern group. Museum abbreviations: SNOMNH, Sam Noble Oklahoma Museum of Natural History; SMNH, Sternberg Museum of Natural History; MVZ, Museum of Vertebrate Zoology; TNHC, Texas Natural History Collection of the Texas Memorial Museum.

Pond Name/ Collection Location	**N**	**Syntopic?**	**GPS latitude (N, decimal degrees)**	**GPS longitude (W, decimal degrees)**	**Population Group**	**Museum Catalog Number**	**Sample source**
Andrews Co., TX	1				C	TNHC 60529	TNHC
Apache (Cochise Co., AZ)	12	Y	31.68	109.14	SW		D. Pfennig
Callaway Co., MO	1		38.59	92.12	C	MVZ 240065	MVZ
Carbon Co., MT	8		45.18	108.91	N	DBS 538, 541, 545, 547, 553, 573, 592, 593	SNOMNH
Cheyenne Co., KS	2				C	MHP 9045, 9046	SMNH
Clark Co., KS	5				C	MHP 9134, 9143, 9145, 9147, 9148	SMNH
Comanche Co., KS	3				C	MHP 8571, 9150, 9151	SMNH
Curry Co., NM	1				C		J. Jones
Custer Co., SD	2				N	MHP 8919, 8921	SMNH
DeBaca Co., NM	3				C		J. Jones

Dickens Co., TX	1				C	TNHC 60526	TNHC
Doniphan Co., KS	1				C	MHP 9098	SMNH
Dunn Co., ND	4		47.38	103.03	N		R. Newman
Dunn Co., ND	5		47.39	103.04	N		R. Newman
Edwards Co., KS	3				C	MHP 9121-9123	SMNH
Ellis Co., OK	3		35.90	99.69	C	DBS 843-845	SNOMNH
Ellis Co., OK	1		36.01	99.76	C	DBS 875	SNOMNH
Four Ten (Cochise Co., AZ)	32	Y	31.74	109.10	SW		D. Pfennig
Grady Co., OK	1		35.25	97.92	C	DBS 391	SNOMNH
Grant Co., KS	1				C	MHP 8994	SMNH
Guy Miller (Cochise Co., AZ)	5	Y	31.88	109.08	SW		D. Pfennig
Harper Co., KS	1				C	MHP 8520	SMNH
Horned Cow (Cochise Co., AZ)	11	Y	31.74	109.10	SW		D. Pfennig
Javelina South (Hidalgo Co., NM)	6	Y	31.82	109.02	SW		D. Pfennig
Johnson Co., KS	2		Near De Soto		C	MVZ 234170, 234171	MVZ
King Co., TX	1				C	TNHC 60525	TNHC

Kingman Co., KS	1				C	MHP 8240	SMNH
Lamb Co., TX	20		Immediate vicinity and south of Springlake		C		R. Martin
Lincoln Co., CO	22		Near Punkin Center		C		D. Pfennig
Logan Co., KS	1				C	MHP 9233	SMNH
McKenzie Co., ND	5		47.40	103.17	N		R. Newman
Meade Co., KS	3				C	MHP 8998, 9015, 9137	SMNH
Otero Co., CO	9		Immediate vicinity of La Junta		C		R. Martin
Payne Co., OK	7		35.99	97.04	C	MVZ 145173-145177, 145205-145206	MVZ
Payne Co., OK	2		Immediate vicinity of Stillwater		C	MVZ 149680, 164812	MVZ
Post Office Canyon (Cochise Co., AZ)	10	Y	31.77	109.08	SW		D. Pfennig
Pratt Co., KS	1				C	MHP 8236	SMNH
Quay Co., NM	2		35.29	103.51	C		J. Jones
Roger Mills Co., OK	3		35.90	99.83	C	DBS 839-841	SNOMNH
Shrimp (Cochise Co., AZ)	6	Y	31.77	109.08	SW		D. Pfennig

Skeleton Canyon (Cochise Co., AZ)	5	Y	31.69	109.11	SW		D. Pfennig
Sky Ranch (Cochise Co., AZ)	4	Y	31.79	109.06	SW		D. Pfennig
Slope Co., ND	4		46.31	103.97	N		R. Newman
Stevens Co., KS	2				C	MHP 8990, 8991	SMNH
Sulfur Draw (Cochise Co., AZ)	20	Y	31.81	109.05	SW		D. Pfennig
Washington Co., CO	20		Immediate vicinity of Last Chance		C		R. Martin
Willcox -Blue Sky (Cochise Co., AZ)	5		32.22	109.78	SW		D. Pfennig
Willcox 11 (Cochise Co., AZ)	11				SW		D. Pfennig
Willcox 12 (Cochise Co., AZ)	7				SW		D. Pfennig
Willcox 13 (Cochise Co., AZ)	11				SW		D. Pfennig
Willcox 8 (Cochise Co., AZ)	33		32.21	109.78	SW		D. Pfennig
Willcox 9 (Cochise Co., AZ)	13		32.21	109.78	SW		D. Pfennig
Winkler Co., TX	1		31.93	103.17	C		S. Lowe

Winkler Co., TX	1		C	TNHC 60528	TNHC

Table A.2. *Spea multiplicata* collection locations and sample sizes. The ten ponds marked as syntopic were included in the IBD analysis presented in the paper.

Pond/Location Name	N	Syntopic?	GPS latitude (N, decimal degrees)	GPS longitude (W, decimal degrees)	County
Apache	17	Y	31.68	109.14	Cochise Co., AZ
Bull Pond	20		31.89	109.10	Cochise Co., AZ
Cholla Pond	19		31.82	109.05	Hidalgo Co., NM
Corner Pond	14		31.86	109.04	Hidalgo Co., NM
Figure Eight	10		31.93	109.09	Cochise Co., AZ
Four Ten	11	Y	31.74	109.10	Cochise Co., AZ
Guy Miller	19	Y	31.88	109.08	Cochise Co., AZ
Horned Cow	13	Y	31.74	109.10	Cochise Co., AZ
Horseshoe	15		31.94	109.09	Cochise Co., AZ
Javelina North	9		31.82	109.02	Hidalgo Co., NM
Javelina South	15	Y	31.82	109.02	Hidalgo Co., NM
John Carron	20		31.93	109.13	Cochise Co., AZ
Peach Orchard 1	36		31.90	109.08	Cochise Co., AZ
Peach Orchard 2	13		31.91	109.08	Cochise Co., AZ
Post Office Canyon	9	Y	31.77	109.08	Cochise Co., AZ
Price Canyon	14		31.67	109.23	Cochise Co., AZ
Rock Tank	20		31.94	109.12	Cochise Co., AZ

Shrimp	19	Y	31.77	109.08	Cochise Co., AZ
Skeleton Canyon	10	Y	31.69	109.11	Cochise Co., AZ
Sky Ranch	5	Y	31.79	109.06	Cochise Co., AZ
Starview	15		31.91	109.13	Cochise Co., AZ
State Line	9		31.85	109.05	Hidalgo Co., NM
Sulfur Draw	8	Y	31.81	109.05	Cochise Co., AZ
Troller	28		31.93	109.12	Cochise Co., AZ
Turkey Creek & Kuykendall Cutoff	11		31.88	109.49	Cochise Co., AZ
Windmill	19		31.87	109.05	Cochise Co., AZ
Yucca Wash	20		31.64	109.09	Cochise Co., AZ

Table A.3. Distribution and occurrence of cytochrome *b* haplotypes from *Spea bombifrons* collection locations. The number of individuals possessing each haplotype is listed in parentheses following each location.

Haplotype	GenBank Accession No(s).	N	Geographic occurrence
1	EU285613, EU499393-EU499416	100	Callaway County, MO (1); Carbon County, MT (8); Cheyenne County, KS (1); Clark County, KS (3); Curry County, NM (1); Custer County, SD (2); DeBaca County, NM (1); Dickens County, TX (1); Dunn County, ND (9); Edwards County, KS (1); Ellis County, OK (1); Johnson County, KS (2); Lamb County, TX (11); Lincoln County, CO (19); McKenzie County, ND (5); Meade County, KS (1); Otero County, CO (8); Payne County, OK (2); Quay County, NM (1); Roger Mills County, OK (1); Slope County, ND (4); Washington County, CO (16); Winkler County, TX (1)
2	EU285614	2	Lamb County, TX (1); Winkler County, TX (1)
3	EU285615	166	Apache (8); Four Ten (22); Horned Cow (11); Javelina South (6); Post Office Canyon (8); Shrimp (6); Skeleton Canyon (2); Sky Ranch (4); Sulfur Draw (18); Guy Miller (5); Blue Sky-Willcox (5); Willcox 8 (33); Wilcox 9 (13); Willcox 11 (10); Willcox 12 (6); Willcox 13 (9)
4	EU285616, EU499418-EU499427	16	Logan County, KS (1); Comanche County, KS (1); Clark County, KS (2); Stevens County, KS (1); Edwards County, KS (1);

			Lincoln County, CO (2); Cheyenne County, KS (1); Washington County, CO (3); Doniphan County, KS (1); Payne County, OK (2); Grant County, KS (1)
5	EU285617	2	Otero County, CO (1); Payne County, OK (1)
6	EU285618, EU499429-EU499435	14	Lamb County, TX (6); King County, TX (1); Meade County, KS (1); Roger Mills County, OK (1); Ellis County, OK (3); Grady County, OK (1); Payne County, OK (1)
7	EU285619	2	Andrews County, TX (1); Quay County, NM (1)
8	EU285620	7	Four Ten (7)
9	EU285621	1	Pratt County, KS (1)
10	EU285622	1	Meade County, KS (1)
11	EU285623	1	Kingman County, KS (1)
12	EU285624	1	DeBaca County, NM (1)
13	EU285625	1	Sulfur Draw (1)
14	EU285626	1	Lincoln County, CO (1)
15	EU285627	1	Washington County, CO (1)
16	EU285628	1	Payne County, OK (1)
17	EU285629	6	Four Ten (3); Post Office Canyon (2); Sulfur Draw (1)
18	EU285630	1	Comanche County, KS (1)

19	EU285631	1	Edwards County, KS (1)
20	EU285632	1	Payne County, OK (1)
21	EU285633	1	Comanche County, KS (1)
22	EU285634	1	Roger Mills County, OK (1)
23	EU285635	1	DeBaca County, NM (1)
24	EU285636	1	Lamb County, TX (1)
25	EU285637	1	Harper County, KS (1)
26	EU285638	1	Lamb County, TX (1)
27	EU285639	1	Payne County, OK (1)
28	EU285640	1	Stevens County, KS (1)
29	EU285641	10	Apache (3); Skeleton Canyon (3); Willcox 11 (1); Willcox 12 (1); Willcox 13 (2)
30	EU285642	1	Apache (1)

Table A.4. Distribution and occurrence of cytochrome *b* haplotypes from *Spea multiplicata* collection locations. The number of individuals possessing each haplotype is listed in parentheses following each location.

Haplotype	GenBank Accession No.	N	Geographic Occurrence
32	EU285643	278	Apache (15); Bull Pond (16); Cholla (14); Corner Pond (10); Four Ten (10); Guy Miller (7); Horned Cow (8); Horseshoe (6); Javelina North (6); Javelina South (12); John Carron (15); Peach Orchard 1 (33); Peach Orchard 2 (8); Post Office Canyon (6); Price Canyon (12); Rock Tank (8); Shrimp (13); Skeleton Canyon (10); Sky Ranch (4); Starview (10); State Line (4); Sulfur Draw (6); Troller (9); Turkey Creek & Kuykendall Cutoff (6); Windmill (12); Yucca Wash (18)
33	EU285644	18	Apache (1); Corner Pond (1); Horseshoe (4); Javelina North (1); Javelina South (3); Rock Tank (5); State Line (1); Troller (2)
34	EU285645	12	Cholla (3); Rock Tank (1); State Line (4); Sulfur Draw (1); Troller (3)
35	EU285646	14	Guy Miller (12); Peach Orchard 1 (1); Troller (1)
36	EU285647	30	Bull Pond (4); Cholla (1); Corner Pond (1); Horned Cow (3); Horseshoe (3); Javelina North (2); Peach Orchard 1 (2); Peach Orchard 2 (1); Post Office Canyon (1); Starview (5); Troller (7)
37	EU285648	22	Corner Pond (1); Peach Orchard 2 (3); Post Office Canyon (1); Price Canyon (1); Rock

			Tank (3); Shrimp (6); Sky Ranch (1); Troller (3); Windmill (1); Yucca Wash (2)
39	EU285649	32	Apache (1); Corner Pond (1); Figure Eight (9); Four Ten (1); Horned Cow (1); Horseshoe (2); John Carron (5); Price Canyon (1); Rock Tank (1); Sulfur Draw (1); Troller (2); Turkey Creek & Kuykendall Cutoff (2); Windmill (5)
40	EU285650	6	Four Ten (1); Rock Tank (2); Troller (1); Turkey Creek & Kuykendall Cutoff (2)
42	EU285651	1	Figure Eight (1)
44	EU285652	1	Peach Orchard 2 (1)
45	EU285653	1	Cholla (1)
46	EU285654	1	Horned Cow (1)
49	EU285655	1	Turkey Creek & Kuykendall Cutoff (1)
50	EU285656	1	Windmill (1)
51	EU285657	1	Post Office Canyon (1)

Table A.5. AMOVA Results for *S. bombifrons*: three population groups (Fig. 3.1).

Source of variation	d.f.	SS	Percentage of variation
Among groups	2	89.77	63.18
Among populations within groups	48	30.27	9.14
Within population	294	59.34	27.68
Total	344	179.38	

F-statistics

$F_{CT} = 0.632$, $P < 0.00001$

$F_{SC} = 0.248$, $P < 0.00001$

$F_{ST} = 0.723$, $P < 0.00001$

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