Behavior, Selection, and the Genetics of Adaptation During the Early Stages of Ecological Divergence

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Authorization to Submit Dissertation

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Abstract

Adaptive divergence is a complex process, the outcome of which is often difficult to predict. The ecological speciation continuum has recently emerged as a framework for understanding the dynamic nature of adaptive divergence and identifying factors that facilitate or inhibit the evolution of reproductive isolation. However, the relative contributions of different factors to progress along the speciation continuum during real-world instances of adaptive divergence remain poorly understood in most systems

The White Sands system provides an opportunity to investigate how different factors influence position along the speciation continuum. White Sands, a gypsum dune field that formed recently within the Chihuahuan desert, represents a striking contrast to the surrounding desert scrubland. Three lizard species have colonized the novel White Sands environment: the Eastern Fence Lizard (*Sceloporus undulatus*), the Little Striped Whiptail (*Aspidoscelis inornata*), and the Lesser Earless Lizard (*Holbrookia maculata*). In all three species, populations on and off White Sands have diverged in a number of phenotypic characteristics and are likely in the incipient stages of ecological speciation.

My dissertation research focuses on how factors such as behavior, selection, and the genetics of adaptation affect the evolution of reproductive isolation during the early stages of ecological speciation, focusing primarily on the White Sands system. In Chapter II I examined mate preference between ecologically distinct populations of *S. undulatus* in White Sands, dark soils, and lava flow habitats and found evidence for asymmetrical reproductive isolation. In Chapter III I investigated selection on color in *H. maculata*, and I found that space, time, and sex influence the dynamics of predation on lizards in White Sands. In Chapter IV I used computer simulations to demonstrate that reproductive isolation evolves more rapidly during ecological speciation when adaptation occurs from standing genetic

variation compared with new mutation. Finally, in Chapter V I explored the relative roles of ecological barriers and geographic distance in generating genetic divergence between populations. I found that White Sands acts as a barrier to gene flow in both species, and that the magnitude of the effect of White Sands on genetic divergence is most likely greater in *S. undulatus* than *A. inornata*.

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Table of Contents

List of Tables

List of Figures

CHAPTER I

Introduction

Speciation is an undeniably complex process, responsible for generating incredible levels of biological diversity throughout our planet's history. As a result of the complicated nature of new species formation, the field of speciation research has seen a number of paradigm shifts, with the framework through which evolutionary biologists study speciation changing dramatically over time. During the Modern Synthesis Ernst Mayr laid the foundation for contemporary speciation research when he proposed the biological species concept, which defines species as reproductively isolated groups of interbreeding populations (1942). Mayr's definition facilitated subsequent speciation research by presenting a measurable benchmark for determining whether populations represent distinct species (Coyne and Orr 2004).

After the Modern Synthesis, speciation research focused to a large degree on identifying the causes of new species formation in nature, typically using the biological species concept as a metric for speciation. One result of this effort was the theory of ecological speciation, where adaptation to distinct environments causes the evolution of reproductive isolation between populations (Schluter 1996). Ecological speciation has three main components: a source of divergent selection between environments, a form of reproductive isolation between populations in different environments, and a genetic mechanism (i.e., linkage disequilibrium or pleiotropy) to link the two (Rundle and Nosil 2005). The idea that divergent selection could lead to speciation dates back to Darwin, but the theory was formalized as compelling evidence of ecological speciation in natural systems emerged in the late 20th century. A number of well-studied examples such as benthic and

limnetic sticklebacks in postglacial lakes (Boughman 2001; Hatfield and Schluter 1999; Nagel and Schluter 1998; Rundle et al. 2000), Mullerian mimicry in *Heliconius* butterflies (Mallet et al. 1998), floral traits and pollinator preference in monkeyflowers (Schemske and Bradshaw 1999), beak size and resource availability in Darwin's finches (Grant and Grant 1992; Grant and Grant 1993; Podos 2001; Ratcliff and Grant 1983), and the timing of diapause in the apple maggot fly (Feder 1998; Filchak 2000), emerged at this time and convinced evolutionary biologists of ecological speciation's significance as a mechanism of new species formation.

But even as evidence for the central role of natural selection in speciation accumulated, the emerging body of ecological speciation research led evolutionary biologists to another important realization- that in some systems where strong divergent selection was present, ecological speciation did not result. In other words, the occurrence of the "ingredients" necessary for ecological speciation was not always sufficient for the evolution of high levels of reproductive isolation. At this time a new perspective on speciation emerged in the literature, as a number of evolutionary biologists suggested that it would be more appropriate to think of speciation not as an endpoint (i.e., complete reproductive isolation between populations), but as a continuous process (Gourbiere and Mallet 2010; Hendry 2009; Mallet et al. 2007; Merrill et al. 2011; Nosil et al. 2009; Peccoud et al. 2009). The ecological speciation continuum is the idea that "progress" towards speciation can be thought of as continuously distributed, from adaptive variation within panmictic populations to complete reproductive isolation between distinct species (Hendry 2009). The ecological speciation continuum is innovative in that it allows researchers to consider a range of potential outcomes of the speciation process. Speciation in the natural world is a spectrum,

with populations exhibiting all imaginable degrees of reproductive isolation, and the ecological speciation continuum reflects that variation.

Moreover, one can consider the position of different populations along the ecological speciation continuum in order to identify and better understand factors that affect progress toward or away from speciation. For example, researchers have investigated the effects of strong versus multifarious divergent selection (e.g., Doebeli and Dieckmann 2003; Fry 2003; Gavrilets 2004; de Leon et al. 2010; Nosil and Sandoval 2008; Nosil et al. 2009; Thibert-Plante and Hendry 2009), the geographic context of speciation (e.g., Doebeli and Dieckmann 2003; Gavrilets et al. 2000; Gavrilets 2004), the dimensionality of ecological shifts (e.g., Funk et al. 2006: Gavrilets 2004; Nosil et al. 2009; Price 2007), and the evolution of assortative mating (e.g., Bolnick and Fitzpatrick 2007; Dieckmann and Doebeli 1999; Doebeli and Dieckmann 2003; Matessi et al. 2001; Gavrilets 2004) in facilitating ecological speciation by studying populations with different levels of reproductive isolation. These studies suggest that progress along the speciation continuum is often complex and difficult to predict. Research that continues to disentangle the web of interacting factors contributing to position along the continuum is a crucial next step towards a complete understanding of ecology's role in generating new species.

The White Sands system provides an opportunity to investigate how different factors influence progress along the speciation continuum. White Sands is a vast dune field that formed within the Chihuahuan desert in New Mexico less than 10,000 years ago (Kocurek et al. 2007). The sparsely vegetated white gypsum sand dunes of White Sands represent a striking contrast to the surrounding desert scrubland environment (referred to here as "dark soils"), which is characterized by brown substrate and a dense distribution of shrubs,

succulents, and grasses. Three lizard species have colonized White Sands from the dark soils environment: the Eastern Fence Lizard (*Sceloporus undulatus*), the Little Striped Whiptail (*Aspidoscelis inornata*), and the Lesser Earless Lizard (*Holbrookia maculata*).

The drastically different White Sands and dark soils environments provide a potential source of strong divergent selection, and previous research suggests that lizards in White Sands have historically been subject to distinct selection pressures compared with their dark soils conspecifics. White Sands and dark soils lizards have diverged in a number of phenotypic characteristics, despite ongoing gene flow between White Sands and dark soils populations (Rosenblum 2006; Rosenblum et al. 2007). Populations in the two habitats exhibit differences in body shape (Des Roches et al. 2013; Rosenblum and Harmon 2011) and behavior (Robertson et al. 2011; Robertson and Rosenblum 2010; Rosenblum 2008), but the most striking phenotypic difference between White Sands and dark soils lizards is body color. In all three species, dark soils individuals exhibit dorsal coloration in shades of brown and black, while their White Sands conspecifics exhibit bright, blanched dorsal coloration (Dixon 1967; Hager 2001; Lowe and Norris 1956; Smith 1943). There is a significant correlation in all three species between the blanched color phenotype and alleles at the *Mc1r* locus (a gene which is known to play a role in determining the density and distribution of melanin in the skin of vertebrates [Barsh 1996]) (Rosenblum et al. 2004), and in two of the three lizard species (*S. undulatus* and *A. inornata*) previous research has identified the functional basis of pigmentation loss via specific *Mc1r* mutations (Rosenblum et al. 2010). Genetic divergence between White Sands and dark soils lizard populations is greater at the *Mc1r* locus than at neutral loci (Rosenblum et al. 2004), indicating that selection on body color has played a role in local adaptation in this system.

Though only recently diverged, lizard populations from the drastically different White Sands and dark soils environments exhibit signs of incipient reproductive isolation. Previous research has demonstrated that both White Sands and dark soils *H. maculata* preferentially court local mates (Rosenblum 2008). It is possible that color may be acting as a magic trait in this system, providing a genetic mechanism to link divergent selection and the evolution of reproductive isolation. All three species of lizards have social signaling coloration used during behavioral interactions, and each species exhibits pronounced differences in signal color between White Sands and dark soils populations (Robertson and Rosenblum 2009). If changes in melanin density and distribution that facilitate colonization of White Sands are responsible for divergence in social signal color phenotype, and if those changes in turn influence mate preference, adaptation to this novel environment could be directly responsible for the evolution of reproductive isolation and eventually lead to speciation (Rosenblum and Harmon 2011).

Parallel but distinct instances of adaptation to White Sands provide a window through which we can view the ecological speciation process in a comparative framework. The degree of adaptive divergence between ecologically distinct populations differs among species, as evidenced by phenotypic and genetic data. Spectrophotometric data indicate that *H. maculata* exhibit the greatest degree of divergence in body color between White Sands and dark soils populations, while *S. undulatus* exhibit intermediate levels, and *A. inornata* exhibit the lowest degree of divergence. Genetic differentiation between White Sands and dark soils populations is concordant with patterns of divergence in body color; genetic clustering analyses based on nuclear and mitochondrial DNA demonstrate strong genetic clustering of *H. maculata* by habitat, intermediate clustering of *S. undulatus* by habitat, and

almost no clustering of *A. inornata* by habitat (Rosenblum 2006; Rosenblum and Harmon 2011).

My dissertation work investigates ecological divergence in the White Sands system, with a focus on understanding how factors such as asymmetrical mate preference, variable selection, demographic structure, and the genetic architecture of traits under selection affect progress towards speciation. In Chapter II I examined mate preference between ecologically distinct populations of *S. undulatus* in White Sands, dark soils, and lava flow habitats. I found evidence for asymmetrical reproductive isolation, where White Sands males preferentially courted local females, but males from other habitats did not. In Chapter III I investigated selection on color in *H. maculata*, and I found that space, time, and sex influence the dynamics of predation on lizards in White Sands. In Chapter IV I used computer simulations to demonstrate that reproductive isolation evolves more rapidly during ecological speciation when adaptation occurs from standing genetic variation compared with new mutation, and that the effect of standing variation is mediated by factors such as migration, mechanisms of reproductive isolation between populations, and mutational covariance between traits. Finally, in Chapter V I explored the relative roles ecological barriers and geographic distance in generating genetic divergence between populations. I found that local adaptation is an important driver of genetic divergence in both species, and that the magnitude of the effect of White Sands as a barrier to gene flow is likely greater for *S. undulatus* than it is for *A. inornata*.

The ecological speciation continuum is a productive framework for studying the complexities of new species formation. Researchers have only just begun to scratch the surface in terms of understanding how different factors affect progress along the speciation

continuum during ecological speciation. In a system as seemingly straightforward as White Sands where evolution is literally black and white, the complexity underlying ecological divergence in these three species of lizards is striking. My dissertation demonstrates the importance of considering speciation as a complex network of interacting factors in order to fully understand ecology's role in generating new species.

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CHAPTER II

Asymmetrical Mate Preference in Recently Adapted White Sands and Black Lava Populations of *Sceloporus undulatus*

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Abstract

Speciation can proceed rapidly when natural and sexual selection act in concert. For example speciation can be accelerated when traits that confer a selective advantage in a particular habitat also influence mate preference. Studying parallel but evolutionarily independent instances of ecological divergence can illuminate the interaction between natural and sexual selection during speciation. Locally adapted populations of the eastern fence lizard (*Sceloporus undulatus*) have recently evolved in three different habitats in the Chihuahuan desert: blanched color morphs occur on the gypsum dunes of White Sands, melanic color morphs occur on the Carrizozo lava flow, and brown color morphs occur in the surrounding desert scrubland. In addition to differences in cryptic dorsal coloration, populations also differ in the size and color of ventral patches used for social signaling. This system therefore provides an opportunity to investigate the interplay of natural and sexual selection during rapid ecological speciation. We used mate preference experiments to determine whether locally adapted populations may exhibit the early stages of behavioral reproductive isolation. We observed an asymmetrical mate preference in this system; white sands males preferentially courted local females, while males from dark soils and black lava populations did not exhibit a preference for local mates. We also found that female behavior and ventral patch phenotype were associated with male courtship. Our results suggest that the

observed preference for local mates evolved at White Sands, and we discuss the possible link between local adaptation and traits involved in mate preference in this system.

Introduction

Understanding the interaction between natural and sexual selection during ecological divergence is a central goal of speciation research (Ritchie 2007; Maan and Seehausen 2011). For populations undergoing divergent selection, mate preference can accelerate reproductive isolation (e.g., Lande 1981; Barraclough *et al.* 1995; Seehausen *et al.* 1997; Price 1998, Boughman *et al.* 2005). In fact, speciation can proceed rapidly when traits subject to divergent natural selection have pleiotropic effects that cause assortative mating (Maynard Smith 1966; Gavrilets 2004). A recent review suggested that traits linking ecology and mating may not be rare in natural populations (Servedio *et al.* 2011), and there are a number of empirical examples of traits that pleiotropically affect adaptation and reproductive isolation in animal populations (e.g., beak morphology in Darwin's finches [Podos and Nowicki 2004], body size in stickleback fish [Nagel and Schluter 1998], color pattern in *Heliconius* butterflies [Jiggins *et al.* 2001]). It is now important to determine how ubiquitous the interaction is between natural and sexual selection during rapid ecological divergence.

Ecologically distinct populations of the eastern fence lizard (*Sceloporus undulatus*) in the Chihuahuan desert of New Mexico represent an ideal system to study the interplay of local adaptation and mate preference. Locally adapted morphs of *S. undulatus* occur in three dramatically different habitats in the Chihuahuan desert. Blanched color morphs occur at White Sands, a habitat with white gypsum substrate. Melanic color morphs occur at the Carrizozo lava flow, a habitat composed of black basalt deposits. Brown morphs are found in

the surrounding Chihuahuan "dark soils" scrubland, a habitat characterized by brown substrate. Dark soils populations of *S. undulatus* are ancestral to white sands and black lava populations (Rosenblum *et al.* 2007). Both White Sands and the Carrizozo lava flow are geologically recent formations of approximately equal size that were likely colonized by *S. undulatus* less than 6,000 years ago (Kocurek *et al.* 2007; Fryberger, unpublished data).

The parallel and rapid evolution of cryptic *S. undulatus* ecotypes in white sands and black lava habitats allows us to evaluate the links between natural and sexual selection in independent but comparable natural evolutionary experiments. Color is a key trait promoting ecological divergence in this system as it plays a role in both predator avoidance and intraspecific interactions (Robertson and Rosenblum 2009). Dorsal coloration is important for reptile crypsis and often evolves rapidly in habitats with different colored substrates. Substrate-matching coloration for *S. undulatus* in the Chihuahuan Desert is presumably an adaptation for avoidance of visually hunting predators such as the greater roadrunner (*Geococcyx californianus*) and the loggerhead shrike (*Lanius ludovicianus*) (Rosenblum 2006). *Sceloporus* lizards also have bright blue ventral color patches which are used for intraspecific communication (Cooper and Burns 1987). Both ventral patch size and color vary across *S. undulatus* populations and may be used as cues for population recognition (Robertson and Rosenblum 2009, 2010). Natural and sexual selection on color may be mechanistically linked in this system because the color of both dorsal and ventral patches is largely determined by the density and distribution of melanin in the skin (Bagnara and Hadley 1973). Therefore changes in melanin production due to natural selection for substrate-matching can have a by-product effect on ventral color patches, potentially impacting mate preference and playing a role in reproductive isolation in this system.

In addition to population differences in coloration, there is evidence that the focal *S. undulatus* populations are in the early stages of ecological speciation (Rosenblum and Harmon 2011). White sands and dark soils populations differ not only in dorsal and ventral coloration but also in other ecologically important morphological (e.g., body shape) and behavioral (e.g., territorial and anti-predator response) traits (Robertson and Rosenblum 2010; Robertson *et al.* 2011; Rosenblum and Harmon 2011). Corresponding ecological studies have not yet compared black lava and dark soils populations, but genetic data suggest some degree of isolation among all three populations (Rosenblum *et al.* 2007).

An important and unanswered question in this system is whether locally adapted populations exhibit behavioral reproductive isolating mechanisms, a critical component in accelerating the process of ecological speciation. Therefore, we tested whether different *S. undulatus* ecotypes exhibit a preference for local mates. Using sequential behavior experiments in males' natural territories, we evaluated mate preferences in one ancestral and two derived populations with independent evolutionary histories. Specifically, we asked whether white sands, dark soils, and black lava *S. undulatus* males preferentially courted local (i.e., ecologically similar) versus foreign (i.e., ecologically distinct) females and determined the extent to which preferences were symmetrical across populations.

Materials and Methods

Data Collection

We conducted mate preference experiments in the field with *S. undulatus*. During the breeding season, adult *S. undulatus* males are highly territorial, and several females may be found within a single male's territory at one time (Haenel *et al.* 2003). Some long-term

association between males and females has been observed (Ferguson 1970), but males will also court novel females (Cooper and Burns 1987; Haenel *et al.* 2003). Individual courtship interactions are generally brief, so behaviors can be scored in relatively short trials in a natural context. We conducted trials from May to July 2010 during the peak of the local *S. undulatus* breeding season (Vinegar 1975; Smith and John-Alder 1999) and during the hours of 07:30–12:00 when males are active. All males in the study were reproductively mature (mean SVL = 5.8 cm). Each male was presented sequentially with one local and one foreign female. This resulted in four trial categories: 1) white sands males presented with white sands vs. dark soils females, 2) dark soils males presented with dark soils vs. white sand females, 3) dark soils males presented with dark soils vs. black lava females, and 4) black lava males presented with black lava vs. dark soils females (Figure 2.1). We used these focal populations because white sands and black lava populations represent independent and recently evolved distinct ecotypes. There were two trial categories for dark soils males because the dark soils population is ancestral to populations in both novel habitats (Rosenblum *et al.* 2007). In each trial category we conducted between 19 and 33 trials for a total of 96 trials (Figure 2.1). We observed male behavior in at least 16 trials per trial category (Figure 2.1).

Prior to behavioral trials, we captured females from the three habitats by hand or noose. We used 18, 20, and 12 reproductively mature females from white sands, dark soils, and black lava habitats respectively. Previous studies of iguanid lizards have shown that males exhibit differences in courtship and territorial behavior towards familiar conspecifics (e.g., Tokarz 1992; Whiting 1999). We therefore collected females from a number of different locations within each habitat to ensure that male courtship response did not merely

reflect familiarity with neighboring individuals. We weighed and measured all females, and took measurements of their dorsal and ventral coloration using a StellarNet EPP2000Cs spectrometer (StellarNet,Tampa, Florida; UV-VIS range of 280 – 900 mm) with a deuterium and tungsten/halogen light source (SL4-DT) and a reflectance probe (R600-8-UV-VIS-SR) fitted with a 45 degree angle tip (RTIP45). In addition, we took digital photographs of female ventral surfaces. Each female was used in an average of 4 trials (90% of the females were used in 1-6 trials, although a few females were used in 8-11 trials). Females were never used in more than 3 trials per day. During the experimental period, females were housed individually in small cages with 12 hour light cycles and fed *ad libitum*. After experimental trials females were released at their point of capture.

The test procedure was as follows. For each trial we captured a male in his natural territory by hand or noose. We immediately placed the male in a circular behavioral arena in his territory and allowed him to acclimate for 5 minutes. The arena was made of metal flashing (diameter = 0.85 m; height = 0.35 m) and the inside was painted light brown to eliminate reflectance. The arena was easy to transport and erect quickly, so the same arena was used for all trials. The male was then presented sequentially with two females (one local and one foreign). Females used in trials were size-matched by snout-vent length and their presentation order was randomized. We introduced the first female to the arena by hand via a small hole in the sand at a point in the arena directly opposite of the male's location and in his line of sight. We scored behavioral interactions for 5 minutes (see below). Next we removed the female by noose and the male was allowed to rest for 5 minutes. We then introduced the second female in the same manner and scored behavioral interactions for 5 minutes.

We recorded each five-minute trial using a digital video camera (Canon FS11, Canon, Lake Success, NY, USA). We recorded male and female behavior in the field and subsequently rescored and verified behavioral observations from the videos. Although some *S. undulatus* behaviors are used in multiple contexts, male precopulatory courtship behavior in this species is highly stereotyped (Cooper and Burns 1987; Martins *et al.* 2005). For males, we recorded the following courtship behaviors: pushups (i.e., leg flexion moving the entire body towards and away from the ground), head bobs (i.e., up-down movements of the head), shudderbobs (i.e., multiple rapid up-down movements of the head), tongue flicks, nips to the female tail and neck, mounts, and copulation attempts. We summarized the male data by calculating the latency to first courtship behavior and the total time spent courting. For females, we recorded the following behaviors: sidlehops (i.e., sideways hopping with back arched), pushups, lateral flattening, approaches to the male, and attempts to escape from the male. We summarized the female data by scoring whether or not each behavior was performed and by calculating the time spent in the two most common behaviors (i.e., pushups and sidlehops). Detailed descriptions of all quantified behaviors can be found in Greenberg 1977.

Statistical Analysis

To understand the dynamics of mate preference in this system we used a series of nonparametric categorical analyses because our behavior data were not normally distributed. Our categorical analyses accounted for the paired nature of the behavior trials (e.g., a single male was presented sequentially with two females). For each pair of trials we assigned a preference for the focal male based on which female was courted faster (shorter latency) and

which female was courted longer (longer total time in courtship). We then tested for associations between male preference and female characteristics (detailed below) using binomial tests. Statistical tests were performed for each trial category shown in Figure 2.1. Statistical analyses were conducted in R (Vers. 0.95.262, R Core Development Team 2011) and JMP (Vers. 9, SAS 2011).

First, we asked whether males preferentially courted local vs. foreign females. Specifically, for each trial category we used binomial tests to determine whether local or foreign females elicited shorter latency and longer total time in courtship (removing trial pairs where there was no male courtship towards either female). If males prefer local females we would expect shorter latency until male courtship and longer total time in courtship for local females compared with foreign females. Additionally, to determine whether the proportion of males that preferred to court local females differed from random expectation, we calculated pairwise (PTI) and global (I_{PTI}) indicators of sexual isolation using the program JMating (Vers. 1.0.8, Carvajal-Rodriguez and Rolan-Alvarez 2006). PTI is the observed number of trials where local mates were preferred, divided by the number of trials where (assuming random mating) we would expect local mates to be preferred. I_{PTI} is the joint isolation index calculated from PTI coefficients (Rolan-Alvarez and Caballero 2000). For both PTI and I_{PTI}, average test statistics, standard deviations, and one-tailed probabilities of rejecting the null hypothesis were determined by resampling 10,000 times both for the observed and for the expected frequencies of pairs (Carvajal-Rodriguez and Rolan-Alvarez 2006).

Second, we asked whether male courtship behavior was correlated with any female behaviors. For each pair of trials, we determined which female spent more or less time

performing the behaviors described above (i.e., sidlehops, pushups, lateral flattening, approaches to the male, and attempts to escape from the male). We then used binomial tests to determine whether males preferentially courted females that spent more or less time displaying each of these behaviors. To determine if there were corresponding population differences in female behavior, we used Pearson's chi-square tests to investigate the relationship between female population and whether or not females engaged in behaviors described above.

Third, we asked about the timing of male and female behavior in the trials, which may indicate whether males assess female behavior during courtship interactions. For each focal male we calculated an average latency to courtship (in seconds). We then used Kruskal-Wallis tests to determine whether latency until male courtship was similar for focal males from different populations. We also used binomial tests to determine whether males or females behaved first more often in trials.

Fourth, we asked about phenotypic differences in female coloration across populations and whether female color was correlated with male behavior. To quantify divergence in coloration across populations we used Endler's segmentation method (Endler 1990) to measure hue, chroma, and brightness over the complete visible spectrum (400-700 nm). In addition, we measured the size of female ventral color patches as the ratio of ventral patch area to the total area of the ventral surface from photographs using ImageJ (NIH 2010). We log transformed patch size data because it was not normally distributed. To characterize female color at a multivariate level, we performed a MANOVA comparing hue, chroma, brightness, and patch size among ecologically distinct populations. We then used one-way ANOVAs to compare each aspect of color separately among female populations. To test for

associations between female color and male behavior, for each pair of trials we determined which female had higher or lower values of hue, chroma, brightness, and patch size. We then performed binomial tests to determine whether males preferentially courted females based on these different aspects of color phenotype.

Our non-parametric approach is warranted by the violation of normality in our behavioral data. But one limitation of this approach is the inability to integrate all of the data in a single model. Therefore we conducted one parametric analysis to incorporate multivariate measures of female color and behavior into linear models explaining male preference. Specifically, we performed principal components analyses with female behavioral data (average time spent doing sidlehops and pushups during trials) and female color data (hue, chroma, brightness, and patch size). We then used linear models to test the effects of male source population, female color, and female behavior on male courtship response. We also evaluated the interactions between male source population and each color and behavior variable. We included male identity as a random effect. The model therefore contained the following explanatory variables: male population, male identity, female color PC 1 (corresponding to dorsal hue and brightness), female color PC 2 (corresponding to ventral patch hue, brightness, and size), female behavior PC 1 (corresponding to average time performing sidlehops), female behavior PC 2 (corresponding to average time performing pushups), and the interaction between male population and each female PC. Given the violation of normality, we focus our data interpretation on the non-parametric tests, but cautiously consider the added insights from the linear models.

Results

Focal male and female lizards engaged in precopulatory behavior in the majority of our trials. Of 96 total trials, we observed male courtship behavior in 72 trials and female behavior in 74 trials. There were copulation attempts in 11 trials (5 of these towards local females and 6 towards foreign females [Fisher's exact test, $P = 0.73$]). In general, we did not observe any order effects (i.e., male behavior did not depend on female presentation order, binomial tests, all $P > 0.05$). There was one exception whereby dark soils males presented with local vs. black lava females exhibited shorter latency until courtship for the second female presented (binomial test, $n = 19$, $P = 0.03$). We also did not observe any effect of female identity on male courtship behavior (i.e., no individual female elicited a disproportionately strong courtship response compared with all other females, Wilcoxon rank-sum tests, all $P > 0.05$).

White sands focal males were the only males in our study to exhibit differences in precopulatory behaviors toward local vs. foreign females. White sands males exhibited shorter latency until courtship for local females (i.e., they courted white sands females more quickly than they courted dark soils females) (binomial test, $n = 16$, $P = 0.04$) (Figure 2.2). White sands males also exhibited longer total time in courtship when presented with local females (binomial test, $n = 16$, $P = 0.01$) (Figure 2.2). Neither dark soils nor black lava males exhibited differences in courtship behavior for local vs. foreign females for any metric (i.e., binomial tests of latency until courtship and total time in courtship, all $P > 0.05$).

Measures of sexual isolation using the program JMating were also consistent with the results presented above. Global measures of sexual isolation were significant for total time in courtship for pairings between white sands and dark soils lizards ($I_{PTI} = 0.38$, $P = 0.03$).

Pairwise indices suggested that white sands males preferentially courted white sands females more often than expected by chance (PTI = 1.62, P = 0.04) and dark soils females less often than expected by chance (PTI = 0.37, P = 0.01). Pairwise indices of isolation were not significant for trials where dark soils males were presented with local (PTI = 1.05, P = 0.87) or white sands (PTI = 0.94, P = 0.81) females, indicating asymmetric sexual isolation. In addition, Global I_{PTI} and pairwise PTI were non-significant (all P > 0.05) for trials with black lava and dark soils lizards, indicating a lack of sexual isolation between these ecologically distinct populations.

Male behavior was correlated with female behavior in some of the trial categories. Most notably, white sands males exhibited longer total time in courtship when paired with females that sidlehopped (Table 2.1). Additionally, we observed a nonsignificant trend where white sands males exhibited shorter latency for females that sidlehopped (Table 2.1).Males did not exhibit differences in courtship correlated with any of the other female behaviors quantified in our study (binomial tests, all P > 0.05).

Females did not exhibit population level differences in most of the behaviors quantified in our study (i.e., sidlehops, lateral flattening, approaches to the male, and attempts to escape from the male, all P > 0.05). The lack of population differences in sidlehop behavior is particularly important because male behavior was correlated with female sidlehop behavior in some trials. The only behavior that did show population differences was female pushups; females from white sands engaged in pushup behavior less than either dark soils or black lava females (Table 2.2).

Whether or not males had an opportunity to assess female behavior may depend on if males initiated courtship before or after females displayed. Latency to courtship was

significantly different for different categories of focal males (Kruskal-Wallis test, $n = 96$, $H(2) = 21.64$, $P < 0.01$). Mean latency to courtship was longer for white sands males than for black lava males (Wilcoxon rank-sum test, $n = 56$, $W = 290$, $P < 0.01$), and was also longer for white sands males than for dark soils males (Wilcoxon rank-sum test, $n = 73$, $W = 157.5$, $P < 0.01$). Further, in trials with white sands males there was no significant difference between whether males or females behaved first (males behaved first in 17 trials while females behaved first in 8) (binomial test, $P = 0.12$). In contrast, males initiated courtship behavior before females in trials with both black lava males (males behaved first in 29 trials while females behaved first in 6) (binomial test, $P < 0.01$) and dark soils males (males behaved first in 57 trials while females behaved first in 9) (binomial test, $P < 0.01$).

Females from distinct populations differed in overall color phenotype, which included dorsal and ventral hue, chroma, and brightness, as well as ventral patch size (MANOVA, DF = 2, $F = 8.95$, $P < 0.01$). Specifically, white sands, dark soils, and black lava females differed in dorsal hue, dorsal chroma, and dorsal brightness (Table 2.2). Females from ecologically distinct populations also differed in ventral patch chroma and ventral patch brightness (but not ventral patch hue, Table 2.2). Finally, female ventral patch size varied across populations (Table 2.2).

Male courtship behavior was associated with female patch size in trials with white sands and dark soils focal male categories. White sands males exhibited shorter latency to courtship for females with large ventral patches (i.e., white sands females), and we also observed a nonsignificant trend where white sands males exhibited longer total time in courtship for females with large ventral patches (Table 2.1). Male behavior did not vary with

respect to any other aspect of female color measured (i.e., dorsal and ventral hue, chroma, and brightness) (binomial tests, all $P > 0.05$).

Linear models (incorporating male population, male individual, female color, female behavior, and interactions between male population and each female variable) showed that male courtship response was predicted by the interaction between female color phenotype and male source population. In the linear models, male source population had a significant effect on male latency to courtship (ANOVA, DF = 2, F = 3.12, P = 0.04) and total time in courtship (ANOVA, DF = 2, F = 4.93, $P < 0.01$). The interaction between male population and female color PC 2 (ventral patch color and size) was also significant for male latency to courtship (ANOVA, DF = 2, F = 4.69, P = 0.01) and total time in courtship (ANOVA, DF = 2, F = 4.20, P = 0.02). The interaction effect is explained by the fact that white sands males preferentially courted females with greater values of color PC 2, while dark soils and black lava males did not.

Discussion

We investigated mate preference in lizard populations undergoing rapid ecological divergence. Specifically, we asked whether male *S. undulatus* from ecologically distinct populations preferentially courted local females. We compared male preference in one ancestral (dark soils) and two derived (white sands and black lava) populations. We found that white sands males preferentially courted local females while dark soils and black lava males did not exhibit differences in courtship behavior based on female locality (Figure 2.2). The observed preference asymmetry suggests the evolution of preference at White Sands. We also found that white sands male preference was associated with several aspects of female

morphology and behavior (Table 2.1). Below, we discuss the possible mechanisms involved in mate preference in this system and the evolutionary implications of the observed preference asymmetry across populations.

Mating Cues

Determining the cues used to identify local mates is important for understanding mechanisms of sexual selection. Reptile courtship interactions can involve a number of different signaling modalities (e.g., visual, chemical, tactile), and manipulative experiments are necessary to test the importance of specific cues and their multimodal interactions (Tokarz 1995). Our mate preference trials did not directly test cues males could use to identify local females. However, male behavior in our experiment was associated with several aspects of female behavior and morphology, providing hypotheses for cues that influence male mate preference in this system.

Female ventral patches are likely one of the most important cues for mate preference in this system. Previous studies have demonstrated that ventral patches are an important mating cue in *S. undulatus* by showing that manipulations of female ventral patch size alter male behavioral response (Cooper and Burns 1987). White sands and dark soils females exhibit dramatic differences in dorsal coloration, ventral patch coloration, and ventral patch size (Table 2.2). Further, white sands male behavior was significantly correlated with female patch size (Table 2.1). In fact, male courtship response was best predicted by the interaction of male population and female ventral patch phenotype. In the White Sands system, color appears to be involved in both adaptation (because cryptic dorsal coloration is important for avoiding predators) and incipient reproductive isolation (because female ventral patch

phenotype predicts male courtship response). Color is often the target of both natural and sexual selection, and there are a number of well-studied examples in other systems of color pleiotropically affecting both adaptation and reproductive isolation (e.g., in walking-stick insects [Jiggins *et al.* 2001], butterflies [Fordyce *et al.* 2002], monkeyflowers [Bradshaw and Schemske 2003], coral reef fish [Puebla *et al.* 2007], and poison-dart frogs [Reynolds and Fitzpatrick 2007]). Therefore further studies are warranted at White Sands to understand the specific effect of female coloration on male mate preference and to evaluate the potential for color to act as a "magic trait" (sensu Servedio *et al.* 2011) in this system.

We found that white sands male courtship was associated with aspects of female behavior, indicating that females may actively influence mate preference in this system with solicitation and/or rejection displays. White sands males were the only males that exhibited a preference for local females, and white sands males exhibited a delayed courtship response relative to dark soil and black lava males (i.e., white sands males had a longer average latency until courtship than other males and their courtship often occurred after females displayed). Thus white sands males may have been better able to evaluate female signals during the beginning of staged behavioral interactions. In addition, white sands male total time in courtship was associated with female sidlehop behavior. The context dependence of female sidlehop behavior is poorly understood in lizards, and additional work is needed to understand the significance of female sidlehops in *S. undulatus* courtship interactions (Greenberg 1977; Kelso and Martins 2007). Our data cannot disentangle whether female sidlehop behavior functions as a trigger for male courtship or a response to it. It is important to note that although females that sidlehopped were courted more extensively by white sands males, sidlehop behavior did not vary between white sands and dark soils females. Therefore,

female behavior alone cannot explain white sands male preference but may complement other cues.

Our study focused primarily on male preference, but it is also important to consider the potential for female choice. There are examples of both male and female mate choice in lizards (e.g., Hews 1990; Tokarz 1992; Olsson 1993). We focused on male preference in this study because male *S. undulatus* courtship displays are highly stereotyped, and males display specific behaviors that occur only in a courtship context (Cooper and Burns 1987; Martins *et al.* 2005). Although females are also behaviorally active during courtship, it is more difficult to ascribe female preference because many behaviors are used in multiple contexts (Cooper and Burns 1987; Martins *et al.* 2005). Future research should more explicitly consider the contribution of female choice in the White Sands system and should explore the expected consequences for reproductive isolation of single-sex versus mutual mate choice.

Mate Preference Asymmetry

Our results suggest an asymmetry in sexual isolation and male mating preferences across our focal *S. undulatus* populations (i.e., white sands males preferred local females while dark soils and black lava males showed no preference). Previous studies with model organisms have found that asymmetrical sexual isolation may occur during population divergence and can occur in either direction (i.e., either the ancestral or the derived population can show a larger degree of isolation) (Kaneshiro 1976; Watanabe and Kawanishi 1979). In these studies, mate preference and population divergence in sexually selected traits are frequently required for isolation asymmetry to evolve (Kaneshiro 1980). Empirical studies have detected isolation asymmetry in nature through observations of mate preference

and copulation attempts between different populations in multiple diverse taxa (e.g., wasps [Bordenstein *et al.* 2000], snakes [Shine *et al.* 2002], salamanders [Arnold *et al.* 1996], and fish [McPhail 1969]).

Our results suggest that preference has evolved at White Sands given that only white sands (but not dark soils or black lava) males exhibited a preference for local mates. It is possible that preference for local mates could prevent maladaptive hybridization and the production of poorly background matched offspring. But why would mate preference be found in the derived white sands population and not the ancestral dark soils population? Mate preference may be more important in the derived population than the ancestral population in a "mainland-island" system with local adaptation (Watanabe and Kawanishi 1979; Kirkpatrick and Servedio 1999). A greater degree of gene flow is expected to occur from regions of high to low population density, which can inhibit small peripheral populations from evolving to their local ecological optima (Garcia-Ramos and Kirkpatrick 1997). White Sands is a small "habitat island" surrounded by dark soils populations, so the swamping effects of gene flow are expected to be more pronounced from dark soils into white sands populations than the reverse. The evolution of mate preference could therefore have facilitated local adaptation in the white sands population. Although demographic processes like migration can facilitate the evolution of asymmetrical preference for local mates, there are alternative ways for isolation asymmetry to arise between ecologically distinct populations (e.g., differences across habitats in the strength of sexual selection on certain traits [Gerhardt 2005; Cocroft *et al*. 2010]). Therefore further work is needed to understand the demographic backdrop and the dynamics of natural and sexual selection at White Sands.

It is important to raise several caveats about the observed mate preference asymmetry and its implications for adaptive evolution of white sands *S. undulatus*. Some studies suggest that mating asymmetries may be transitory phenomena observed at intermediate stages of divergence (Arnold *et al.* 1996). Other studies have demonstrated that mating asymmetry may inhibit speciation because, in the context of reinforcement, reproductive isolation is more likely to evolve when gene flow occurs in both directions (Servedio and Kirkpatrick 1997). However, these caveats may not be particularly relevant to the White Sands system. White Sands is a geologically young formation and *S. undulatus* across the ecotone are in the early stages of divergence. Given the recent timeframe for divergence, the observed asymmetry is unlikely to be a transitory phenomenon attributable to intermediate stages of divergence. In addition, theoretical predictions predicated on reinforcement models may not be applicable given that divergence across the White Sands ecotone has occurred in parapatry (rather than allopatry and subsequent recontact). Further work is needed to reconcile theoretical predictions and empirical results in specific case studies like White Sands.

The preference we observed for local mates in one derived population (white sands) was not exhibited by another derived population (black lava). One possible explanation for this observation is that color may be a more direct link between naturally and sexually selected traits in the white sands population compared to the black lava population. The genetic basis of the derived white sands phenotype is controlled by the melanocortin-1 receptor gene (*Mc1r*; Rosenblum *et al.* 2010), while the melanic lava flow phenotype is not due to a mutation at *Mc1r* (Rosenblum *et al.* 2004). It is possible that the genetic architecture of the melanic phenotype (which remains to be determined) or the melanic phenotype itself provides less of an opportunity for sexual selection. For example, the difference in ventral

patch size is larger between dark soils and white sands females than between dark soils and black lava females. Thus if patch size is a phenotypic cue used to inform mate choice, it is possible that white sands males could more easily discriminate local vs. foreign females than black lava males. Finally, it is possible that black lava or dark soils males do have subtle preferences that were not detected using our metrics or with the geography of our sampling. For example, we used dark soils lizards from a nearby (but allopatric) locality where we could reliably sample large numbers of lizards. Conducting trials with additional populations would be important to confirm a lack of preference for local females in parapatric dark soils and black lava populations.

Conclusion

We examined male mate preference in multiple populations undergoing rapid ecological divergence. We found evidence for mate preference at White Sands, whereby males favored local females. Preference for local mates was associated with female ventral patch phenotype, which may indicate that color is playing a role pleiotropically in both adaptation and reproductive isolation in this system. The finding of mate preference in white sands *S. undulatus* provides behavioral evidence that white sands lizards are undergoing the early stages of ecological speciation (Rosenblum and Harmon 2011). Rosenblum (2008) previously demonstrated a preference for local mates in another species with a white form at White Sands, *Holbrookia maculata* (Rosenblum 2008). That two White Sands species exhibit a preference for local mates after only several thousand years of divergence suggests that sexual selection may play a key role even in the early stages of ecological divergence. However, our results also suggest that mate preference does not necessarily evolve in a

predictable manner in cases of ecological divergence. We detected a preference for local mates in only one of the two derived populations we investigated, indicating that migration-selection balance may be sufficient to maintain the adaptive phenotype in the absence of behavioral isolation. It would be fruitful to compare the degree of genetic isolation for the two derived populations relative to the ancestral population to determine whether the evolution of mate preference at White Sands is associated with accelerated speciation. Lastly, we observed a mate preference asymmetry, whereby the ancestral dark soils population did not exhibit a preference for local mates. Mate preference asymmetry has been observed in other taxa, but additional work is needed to understand how isolation asymmetries may promote or hinder speciation. White Sands represents a fruitful system to further study the interaction between local adaptation, mate preference, and isolation asymmetry during ecological divergence.

Tables

Table 2.1: Effects of female behavior and female color on male courtship response for each trial category (white sands focal males presented with local and dark soils females, dark soils focal males presented with local and white sands females, dark soils focal males presented with local and black lava females, and black lava focal males presented with local and dark soils females). For each set of trials we determined focal male preference based on which female was courted faster (shorter latency) and which female was courted longer (longer total time in courtship). We then tested for associations between male preference and female behavior (time spent performing sidlehops and pushups) and female color (ventral patch size, ventral patch brightness, and dorsal brightness) using binomial tests. Significant results ($P < 0.05$) in bold. Focal male and female populations are indicated by the following abbreviations: sands (white sands), soil (dark soils), and lava (black lava).

		Latency to Courtship					Total Time in Courtship				
		Female Behavior		Female Coloration			Female Behavior		Female Coloration		
Focal Male	Females	Sidlehop	Pushup	Dorsal Brightness	Patch Brightness	Patch Size	Sidlehop	Pushup	Dorsal Brightness	Patch Brightness	Patch Size
Sands	Sands, Soil	0.06	1.00	0.27	0.58	**0.02**	**0.01**	1.00	0.09	1.00	0.09
Soil	Soil, Sands	1.00	0.22	1.00	1.00	1.00	1.00	0.69	1.00	1.00	0.63
Soil	Soil, Lava	0.73	0.12	0.81	0.48	0.24	0.51	1.00	0.65	0.34	0.36
Lava	Lava, Soil	1.00	0.51	0.61	1.00	1.00	1.00	0.75	0.45	0.21	0.45

Table 2.2: Population level differences in female behavior and female coloration. We used Pearson's chi-square tests to determine whether sidlehop and pushup behavior varied among female populations. We also used ANOVAs to determine whether dorsal and ventral hue, chroma, and brightness, as well as ventral patch size, varied among female populations. For traits that varied among female populations, we performed post-hoc tests to determine which pairs of populations differed. All results are reported as test statistic: P-value. Significant results ($P < 0.05$) in bold. Female populations are indicated by the following abbreviations: sands (white sands), soil (dark soils), and lava (black lava).

	Female Behavior		Female Dorsal Coloration			Female Ventral Coloration			
Females Compared	Sidlehop	Pushup	Dorsal Hue	Dorsal Chroma	Dorsal Brightness	Patch Hue	Patch Chroma	Patch Brightness	Patch Size
All Populations	2.02: 0.36	13.55: **<0.01**	24.34: **<0.01**	35.64: **<0.01**	80.68: **<0.01**	2.15: 0.13	3.51: **0.04**	8.95: <**0.01**	10.67: **<0.01**
Pairwise Differences	Sands≈Soil ≈Lava	Sands<Soil, Lava	Sands>Soil> Lava	Sands<Soil, Lava	Sands>Soil> Lava	Sands≈Soil≈ Lava	Sands>Lava Soil≈Sands, Lava	Sands<Soil, Lava	Soil<Sands, Lava

Figures

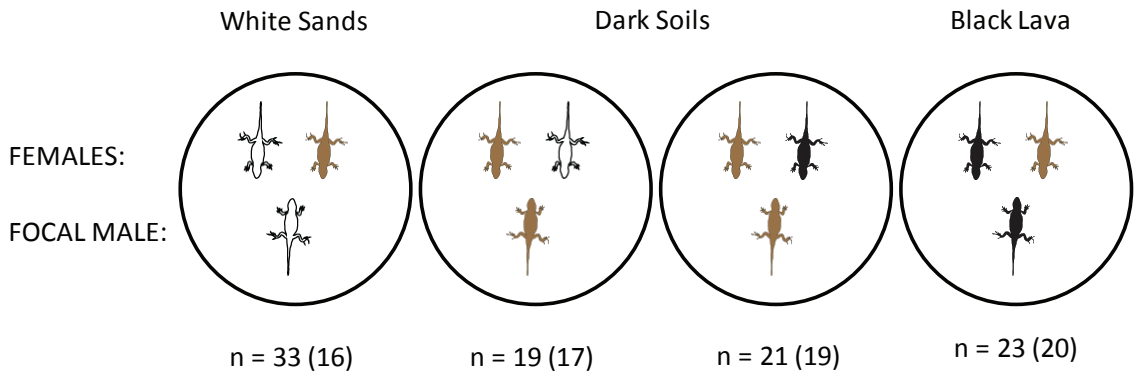

Figure 2.1: Staged arena encounters occurred in the natural territory of white sands (white), dark soils (brown), and black lava (black) focal males. We used a sequential mate preference design to examine the response of each focal male to both local and foreign females. The number of total trials conducted for each category is provided with the number of trials with male behavior in parentheses.

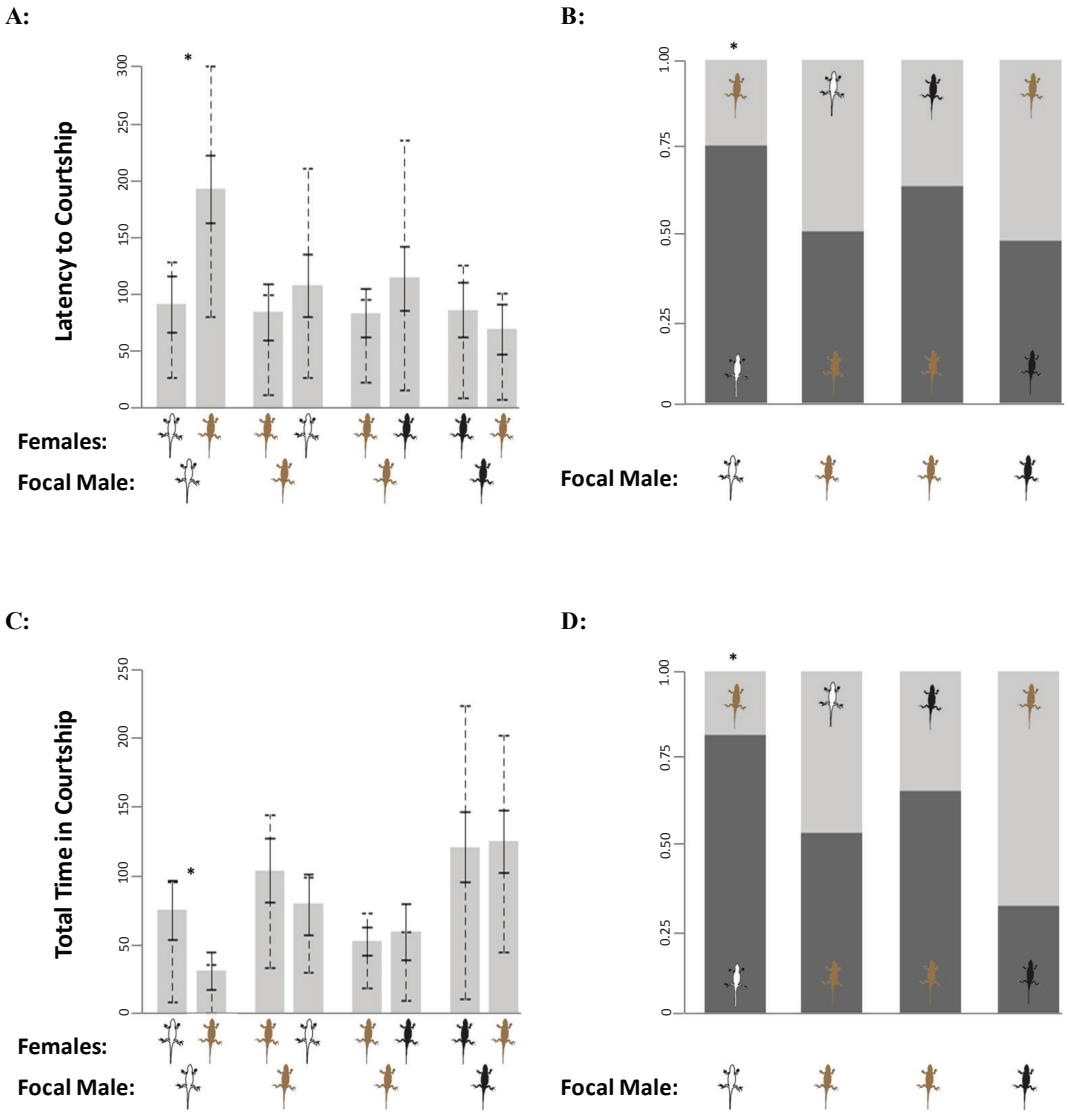

Figure 2.2: Male courtship response to ecologically distinct females in each trial category. "*" indicates a significant ($P < 0.05$) difference in courtship response towards local and foreign females. (A) Mean latency to courtship in seconds by focal males in response to local and foreign females. Error bars with solid lines display standard errors, and error bars with dashed lines display interquartile ranges. (B) Proportion of trial pairs where focal males displayed shorter latency to courtship for local and foreign females. Dark grey bars represent trials with shorter latency for local females, and light grey bars represent trials with shorter latency for foreign females. (C) Mean total time in courtship in seconds by focal males in response to local and foreign females. Error bars with solid lines display standard errors, and error bars with dashed lines display interquartile ranges. (D) Proportion of trial pairs where focal males displayed longer total time in courtship for local and foreign females. Dark grey bars represent trials with longer time in courtship for local females, and light grey bars represent trials with longer time in courtship for foreign females.

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CHAPTER III:

When Field Experiments Yield Unexpected Results: Lessons Learned From Measuring Selection in White Sands Lizards

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Abstract

Determining the adaptive significance of phenotypic traits is key for understanding evolution and diversification in natural populations. However, evolutionary biologists have an incomplete understanding of how specific traits affect fitness in most populations. The White Sands system provides an opportunity to study the adaptive significance of traits in an experimental context. Blanched color evolved recently in three species of lizards inhabiting the gypsum dunes of White Sands and is likely an adaptation to avoid predation. To determine whether there is a relationship between color and susceptibility to predation in White Sands lizards, we conducted enclosure experiments, quantifying survivorship of *Holbrookia maculata* exhibiting substrate-matched and substrate-mismatched phenotypes. Lizards in our study experienced strong predation. Color did not have a significant effect on survival, but we found several unexpected relationships including variation in predation over small spatial and temporal scales. In addition, we detected a marginally significant interaction between sex and color, suggesting selection for substrate matching may be stronger for males than females. We use our results as a case study to examine six major challenges frequently encountered in field-based studies of natural selection, and suggest that insight into the complexities of selection often results when experiments turn out differently than expected.

Introduction

Natural selection plays a central role in shaping patterns of diversification in natural populations (Dobzhansky 1951; Funk 1998; Mayr 1947; Schluter 2001), and is thus a major focus of the field of evolutionary biology. In order to understand how adaptation to distinct environments can result in population differentiation and ultimately speciation, researchers must accurately identify phenotypic targets of divergent natural selection (Schluter 2009). Trait differences between populations are often assumed to have evolved via natural selection (Gould and Lewontin 1979), representing initial stages of ecological speciation. However, direct evidence of specific traits conferring a fitness advantage to locally adapted individuals in natural populations is difficult to obtain and thus somewhat uncommon (e.g., Haller and Hendry 2014, but see Kingsolver et al. 2001). As a result, evolutionary biologists currently have an incomplete understanding of the adaptive significance of specific traits in most systems.

White Sands is an ideal system for taking an experimental approach to understanding how selection on specific traits facilitates adaptation to distinct environments. The area is characterized by white gypsum sand dunes that formed less than 10,000 years ago (Kocurek et al. 2007). In contrast, the surrounding "dark soils" desert scrubland is characterized by brown substrate. Three lizard species are found in both the dark soils and White Sands habitats: *Holbrookia maculata* (the Lesser Earless Lizard), *Sceloporus cowlesi* (the Eastern Fence Lizard), and *Aspidoscelis inornata* (the Little Striped Whiptail). For all three species of lizards, populations in dark soils exhibit brown dorsal color, while populations in White Sands have blanched dorsal color (Dixon 1967; Hager 2001; Lowe and Norris 1956; Smith 1943). The evolution of blanched color in White Sands is most likely an adaptation to avoid

detection by visually oriented avian predators such as *Lanius ludovicianus* (the loggerhead shrike) and *Geococcyx californianus* (the greater roadrunner), both of which occur in White Sands (Kaufman 1973; Raitt and Pimm 1976; Reid and Fulbright 1981).

A number of lines of evidence indicate that blanched dorsal color is adaptive in White Sands lizards. First, there is a correlation between color phenotype and the substrate environment, despite ongoing gene flow (Rosenblum et al. 2007). Second, there is convergence among multiple taxa in the evolution of blanched color in White Sands populations (Bugbee 1942). A number of vertebrate and invertebrate species exhibit blanched color in White Sands populations and a darker dorsal phenotype in dark soils populations, including the three species of lizards mentioned above as well as *Perognathus gypsi* (the Apache pocket mouse) (Dice 1929), *Scaphiopus couchii* (Couch's Spadefoot Toad) (Stroud 1949), and *Daihinoides hastiferum* (the White Sands camel cricket) (Strobecker 1947). Third, analyses of molecular data suggest that *Mc1r*, which plays a role in determining the density and distribution of melanin in the skin of vertebrates (Barsh 1996), has been under divergent selection in this system. There is a significant correlation between the blanched color phenotype and alleles at the *Mc1r* locus in all three White Sands lizard species (Rosenblum et al. 2004), and in two of the three lizard species (*S. cowlesi* and *A. inornata*) the functional basis of pigmentation loss via specific *Mc1r* mutations has been identified (Rosenblum et al. 2010). Genetic divergence between White Sands and dark soils lizard populations is greater at the *Mc1r* locus than at neutral loci (Rosenblum et al. 2004), indicating that selection on body color has played a role in local adaptation in this system.

In light of previous research suggesting there has been selection on dorsal color at White Sands, we sought to experimentally investigate the adaptive significance of blanched

color. Specifically, the objective of our study was to determine whether there is a relationship between body color and susceptibility to predation in White Sands *H. maculata*. We conducted enclosure experiments with *H. maculata* exhibiting substrate-matched and substrate-mismatched color phenotypes (i.e., phenotypes exhibited by White Sands and dark soils lizards, respectively) and quantified survivorship from predation. We report results from our primary objective (assessing selection on color), and also report several unexpected findings that suggest selection at White Sands may be more complex than previously considered.

Materials and Methods

Study Design

Animal care and use protocols for all experiments were approved by the University of California Berkeley ACUC (R347) and the University of Idaho IACUC (2009-37), and permits for field work were approved by White Sands National Monument, White Sands Missile Range, and the New Mexico Department of Game and Fish. We performed enclosure experiments with *H. maculata* individuals, because *H. maculata* exhibit the highest degree of divergence in body color between White Sands and dark soils individuals of the three White Sands lizard species (Rosenblum 2006). In addition, they are likely the most reliant on substrate matching of the three species. *H. maculata* are sit and wait predators (while *A. inornata* are active foragers [Barbault and Maury 1981]) and are less closely associated with vegetation than *S. cowlesi* (Hager 2001). *Holbrookia maculata* individuals in White Sands are active during the months of May through October from 0700 to 1900 hours (with peaks in

activity occurring daily from 1000 to 1400 hours) (Hager 2001), and we conducted trials from May through July in 2011 and 2012.

In 2011 we built three enclosures on the White Sands ecotone, each 100 square meters (10 meters by 10 meters). The ecotone is a narrow band of transitional habitat at the edge of the White Sands formation with light gypsum substrate but a higher density of vegetation than is found in the heart of the dunes. The ecotone was the optimal location to conduct our study because predator densities are also higher in the ecotone than in the heart of the dunes (Des Roches et al. 2011), and because ecotone *H. maculata* are blanched and indistinguishable in color from those found in the heart of the dunes (Rosenblum 2006). While the substrate color of the ecotone is more variable (Rosenblum 2006), we built enclosures in regions where sand color was comparable to that of the heart of the dunes. The mean distance between enclosures was 113.23 meters.

We constructed the enclosures using steel flashing (0.5 meters in height with approximately 15 centimeters buried under the surface of the sand). To anchor flashing in place we fastened it to rebar posts (1.2 meters in height with approximately half of each post buried under the surface) using zip ties. We divided each enclosure in half with a 10 meter long piece of flashing, and covered seams using aluminum foil tape. In 2011, one half of each enclosure allowed avian predators to enter freely (the "open" treatment), and we covered the other half with chicken wire to exclude predators (the "closed" treatment) (Figure 3.1). To create the closed treatment, we used zip ties to fasten chicken wire to the top edge of the steel flashing for one half of each enclosure. To ensure lizards would have an ongoing supply of food we used chicken wire with holes 2.54 centimeters in diameter, which allowed invertebrates to enter and exit freely. To support the chicken wire we placed a

number of rebar posts throughout the closed sides of enclosures, and covered support posts with PVC pipe so that the lizards could not use supports to escape. To make sure lizards had adequate ground cover to seek refuge from biotic and abiotic elements of the environment, we chose enclosure locations where existing vegetation covered approximately 20% of the available space (previous research indicates that White Sands *H. maculata* prefer habitat where on average 20.5% of the ground is covered by vegetation [Hager 2001]). We ensured that our enclosure design was sufficient to contain lizards by conducting brief (15 minute) observations of *H. maculata* within enclosures before initiating trials.

We captured *H. maculata* individuals by hand or by noose from several geographically proximate locations throughout White Sands National Monument (with a maximum distance of nine kilometers between locations) to ensure that we did not impact any one subpopulation disproportionately. Our prior studies have not shown differentiation in behavior, morphology, or genetics among sampling localities within White Sands (Rosenblum and Harmon 2011). To control for any subtle differences among lizards collected from different localities, we randomly assigned lizards to experimental treatments. We recorded sex and took measurements of mass, snout-vent length, and tail length for all individuals. Morphological data for individual lizards are available in the Dryad repository (doi: 10.5061/dryad.068b6). We included adult and subadult lizards in our trials in a body size range of 3.4 to 6.1 centimeters snout-vent length. We excluded juvenile lizards given the potential for predation by larger conspecifics during the course of the experiment. We randomly assigned lizards of different sizes to different treatments and enclosures. We used a black permanent marker to record an individual identification number on the ventral surface of each lizard. Before and after trials, we housed lizards individually in small cages with 12-

hour light cycles. We kept lizards in captivity up to 10 days before the beginning of trials, and released them at their original points of capture no more than one week after trials were completed. While lizards were in captivity we fed them *ad libitum*.

To test whether dorsal color had an effect on lizard survivorship in White Sands, we painted *H. maculata* dorsal surfaces to be substrate-matched or substrate-mismatched (i.e., to represent the color of White Sands or dark soils lizards, respectively) using human temporary tattoo paint (Amunez International) (Figure 3.1). Previous research has shown that tattoo paint can be used to alter the color of lizards for extended periods of time without harmful effects (Olsson et al 2005). We chose to use painted lizards in our experiment rather than lizards captured from dark soils because we were interested in specifically investigating the role of body color in survival. Previous research on the White Sands system has shown that lizards in White Sands differ from those in dark soils in a number of characteristics besides color, including aspects of morphology and behavior (Des Roches et al. 2011; Hardwick et al. 2013; Robertson et al 2011). By using lizards exclusively from White Sands, we controlled for potentially confounding characteristics that differ between populations and were able to examine the effects of color specifically; we were also able to avoid unintentionally releasing non-native individuals into White Sands populations and prevent breeding between White Sands and dark soils individuals.

To obtain paint colors that corresponded to White Sands and dark soils dorsal color, we used spectrometer readings of *H. maculata* dorsal surfaces taken during current and previous field seasons (n = 49 *H. maculata* from White Sands, and n = 23 *H. maculata* from dark soils) (Rosenblum 2006). We determined for both the substrate-matched and substrate-mismatched phenotypes the ratios of white, black, and brown paints that, when mixed and

applied to the dorsal surfaces of White Sands lizards, produced absorbance curves where spectrometer readings fell within the minimum and maximum absorbance values observed at each wavelength from 300-800 nanometers (which encompasses the range of the spectrum visible to most birds [Cuthill et al. 2000]) for White Sands and dark soils lizards. Finally, we painted all lizards to be used in enclosure trials, randomly assigning each individual to either the substrate-matched or substrate-mismatched paint treatment. We took digital photographs of dorsal surfaces before and after painting for all individuals. In addition, for a subset of individuals (n = 24 from the substrate-matched treatment and n = 25 from the substrate-mismatched treatment) we took measurements of dorsal and ventral coloration before and after painting using a StellarNet EPP2000Cs spectrometer (StellarNet,Tampa, Florida; UV-VIS range of 280 – 900 nanometers) with a deuterium and tungsten/halogen light source (SL4-DT) and a reflectance probe (R600-8-UV-VIS-SR) fitted with a 45 degree angle tip (RTIP45). Spectrometer data for painted and unpainted lizards collected in 2011 and 2012 are available in the Dryad repository (doi: 10.5061/dryad.068b6).

In order to ensure that the paint treatments did not interfere with the ability of lizards in the study to thermoregulate, we assessed thermal preference using 20 White Sands *H. maculata.* Half of the individuals used to measure thermal preference were painted to be substrate-matched, and the other half were painted to be substrate-mismatched. Equal proportions of males and females were used in each paint treatment group. To assess preference we filled a 37.9 liter terrarium with White Sands substrate. We placed a light source at one end so that a gradient of substrate temperatures ranging from 27 to 45 degrees Celsius was available within the terrarium. We housed each lizard at room temperature without a heat lamp immediately prior to assessing thermal preference, and then placed the

lizard in the center of the terrarium and allowed it to acclimate for 30 minutes. We took measurements of temperature with a cloacal probe at 30 minute intervals over a 90 minute period, resulting in data collection points at 30, 60, and 90 minutes. At each time point we also recorded the distance of the lizard from the terrarium light source. Thermal preference data are available in the Dryad repository (doi: 10.5061/dryad.068b6). We collected data at multiple time points to ensure that lizards in different paint treatments did not differ in their ability to maintain preferred body temperature over time. We performed assessments of thermal preference on May 31, 2011 and June 1, 2011.

After processing and painting, we released lizards into enclosures (Figure 3.1). We gave all lizards a substantial meal (two medium crickets or mealworms) the evening prior to being released into enclosures. Immediately before initiating trials, we ensured enclosures were empty of lizards and other vertebrates by checking visually and thoroughly raking the sand. We included 14 lizards in each half enclosure, seven from the substrate-matched treatment group and seven from the substrate-mismatched treatment group. We assigned individuals randomly to enclosure treatment, while ensuring that the proportions of males and females in matched and mismatched treatments were consistent across open and closed sides of each enclosure. In 2011, we included all lizards from the thermal preference assessment in a single enclosure trial and randomly assigned them to the open or closed treatment group.

We started the first enclosure trial on June 1, 2011, and staggered start dates for subsequent trials by several days each. By staggering start dates, we were able to increase replication without building additional enclosures, and were also able to run trials over a greater proportion of the activity season. Once a trial was initiated, we visited enclosures

frequently (once every two to three days, or up to three times a day in inclement weather) to ensure structural integrity of the enclosures. During trials we also checked enclosures every several days for signs of visitation by possible predators (i.e., tracks, scat, feathers, owl pellets, hair, shed skin, or lizard carcasses). During enclosure checks we removed any individuals that had shed their skin and transported them to the field station to repaint them. We released repainted individuals into their original enclosures within 24 hours of removing them. We captured survivors from both open and closed sides of enclosures after 16 days. At the end of trials, we recorded the mass of surviving lizards and checked for instances of tail autotomy.

For trials in 2012 we used a total of four enclosures, which included the three constructed in 2011, plus an additional enclosure built in a nearby location in the White Sands ecotone in May 2012. Because we had 100% survivorship of lizards in the control treatment in 2011 (see Results), we did not repeat the predator exclusion portion of the study in 2012. We removed chicken wire from all previously built units so that we had four 100 square meter enclosures, each divided in half for a total of eight open treatment replicates. We included 14 lizards in each half enclosure, seven from the substrate-matched treatment group and seven from the substrate-mismatched treatment group. We staggered start dates for enclosures by several days each, with the earliest starting on May 30. For two enclosures we performed an additional round of enclosure trials after recapturing the first set of survivors, using the same methods with new lizards. We ran a total of three open treatment replicates (n = 42 lizards) and three closed treatment replicates (n = 42 lizards) among three enclosures in 2011, and in 2012 we ran a total of 12 open treatment replicates (n = 168 lizards) among four enclosures. Thus the total number of open treatment replicates for 2011 and 2012 together

was 15 (n = 210 lizards). Recapture data for individual enclosure replicates are available in the Dryad repository (doi: 10.5061/dryad.068b6).

Statistical Analysis

To evaluate how well paint color treatments corresponded to the natural color of White Sands and dark soils lizards, we compared the brightness of painted lizards to that of the White Sands and dark soils lizards used to generate the paint treatments. Brightness is the component of color that accounts for 90% of variation between *H. maculata* from White Sands and dark soils (Rosenblum 2006), and is therefore a good indication of the degree to which paint treatments represent the color of lizards from the two distinct habitats. We used Endler's segmentation method (Endler 1990) to measure brightness over the wavelength range of 300-800 nm for painted and unpainted lizards, and then compared brightness of White Sands and dark soils lizards to that of the corresponding paint treatment using t-tests. To determine whether paint treatment differentially affected the ability of lizards to thermoregulate, we performed ANCOVAs comparing both body temperature and distance from the light source between paint treatments, with time as a covariate.

To understand the dynamics of predation in our enclosure trials, we compared survivorship of lizards with respect to enclosure treatment and paint color treatment. We used Fisher's exact tests to compare the number of surviving lizards between open and closed enclosure treatments for trials conducted in 2011. In addition, we used chi-square contingency tests to compare (within open enclosures) the number of surviving lizards in substrate-matched and substrate-mismatched paint color treatments for trials from 2011 and 2012. Finally, we fit a general linear model with a binomial link function to the open

enclosure data to test the effects of paint treatment, enclosure location, and year on survivorship. Specifically, our response variable was lizard survivorship (yes or no), and our explanatory variables were paint treatment (matched or mismatched), enclosure (one of four possible geographic locations within the ecotone), year (2011 or 2012), sex, and snout-vent length. In addition, we included pairwise interactions between each of these terms in our model. Including enclosure as a factor in our model allowed us to account for the fact that replicates in the same enclosure experience the same microhabitat. We conducted all statistical analyses in R version 2.15.2 (R Development Core Team 2012).

Results

Paint treatments accurately reflected natural colors and did not differentially impact the ability of lizards to thermoregulate. Lizards from the substrate-matched paint treatment did not differ significantly from White Sands *H. maculata* in dorsal brightness ($t_{45.79} = -1.61$, $P = 0.11$), and the same was true for substrate-mismatched lizards and dark soils *H. maculata* ($t_{45.99} = -1.15$, $P = 0.25$). Substrate-matched lizards were significantly brighter than substrate-mismatched lizards ($t_{45.79} = 16.41$, $P < 0.01$), which is consistent with differences between the naturally occurring White Sands and dark soils phenotypes. In addition, paint treatment groups did not differ significantly in average body temperature ($F_{1, 333} = 0.0$, $P = 1.0$) or distance from the light source ($F_{1, 11928} = 0.13$, $P = 0.72$*),* and these patterns were consistent over the course of the entire trial time period ($P = 0.33$ for body temperature; $P = 0.50$ for distance from light source). Lizards utilized in the thermal preference portion of the study did not suffer decreased survivorship in the enclosure trials; survivorship in the open side of the

enclosure with thermal preference lizards in 2011 was 43%, while survivorship in replicates in the same enclosure in 2012 was 41% (95% CI [0%, 85%]).

Survivorship differed significantly between open and closed treatments in trials conducted in 2011, where survivorship was higher for lizards in closed treatments than in open (Fisher's exact test, $P < 0.01$). In fact, 100% of the lizards within the closed treatment group survived, compared with only 36% (95% CI [5%, 67%]) of lizards in the open treatment group (Figure 3.2). However, survivorship in open replicates did not differ between paint treatments ($\chi^2_1 = 0.02$, $P = 0.89$). 36% of lizards in the open treatment survived in 2011, with 38% (95% CI [0%, 100%]) of substrate-matched lizards surviving, and 33% (95% CI [13%, 53%]) of substrate-mismatched lizards surviving. In 2012, 61% (95% CI [42%, 80%]) of lizards survived, with 59% (95% CI [37%, 81%]) of substrate-matched lizards surviving, and 63% (95% CI [46%, 80%]) of substrate-mismatched lizards surviving. Thus for 2011 and 2012 combined, 56% (95% CI [40%, 72%]) of all lizards in the open treatment survived, with 55% (95% CI [36%, 74%]) of substrate-matched lizards surviving and 57% (95% CI [42%, 72%]) of substrate-mismatched lizards surviving (Figure 3.2).

The results of our general linear model indicated that lizard survivorship did not vary with respect to paint treatment ($\chi^2_1 = 0.037$, $P = 0.85$), but did vary with respect to enclosure location and year. Survivorship was significantly higher in trials in 2012 compared with trials in 2011 ($\chi^2_1 = 4.46$, $P = 0.03$) (Figure 3.3). In addition, survivorship for trials in 2011 and 2012 differed dramatically among enclosures ($\chi^2_3 = 37.58$, $P < 0.01$); two enclosures exhibited higher survivorship with 67% (95% CI [5%, 100%]) and 86% (95% CI [62%, 100%]) of lizards recaptured, and two enclosures exhibited lower survivorship with 41% (95% CI [11%, 71%]) and 31% (95% CI [11%, 51%]) of lizards recaptured (Figure 3.3). Our

model detected a significant interaction between enclosure and year ($\chi^2_2 = 7.11$, P = 0.03), where survivorship in some enclosures differed significantly between years and survivorship in other enclosures did not (Figure 3.3). We also observed a marginally significant interaction between paint treatment and sex ($\chi^2_1 = 3.64$, P = 0.05). Mean survivorship of mismatched males was 48% (95% CI [28%, 68%]), and survivorship of matched males was 58% (95% CI [38%, 78%]). The opposite pattern occurred in females, with 67% (95% CI [53%, 81%]) of mismatched individuals surviving, compared with 50% (95% CI [27%, 73%]) of matched individuals (Figure 3.4). However, posthoc tests comparing survivorship between treatments for each sex individually were not statistically significant (Fisher's exact tests, P = 0.23 for males and P = 0.12 for females). Finally, lizard size (i.e., snout-vent length) did not have a significant effect on survivorship ($\chi^2_1 = 2.94$, P = 0.09).

Mass of surviving lizards was significantly lower after trials than it was at the beginning of trials (t-test, $t_{257.79} = -5.57$, P < 0.01), likely reflecting the effects of food limitation and stress due to the high lizard density in enclosures. However, loss of mass throughout trials was similar for substrate-matched and substrate-mismatched individuals (t-test, $t_{128.17} = 0.20$, P = 0.84), indicating that lizards in different paint treatments were not differentially affected. We did not observe any instances of tail autotomy throughout the experiment.

Over the two summers that we conducted the study, we found evidence of avian predators (i.e., tracks, scat, or visual observations of the birds themselves) near enclosures on 29 occasions (18 of those being five or fewer meters from the enclosure walls) and within enclosures on nine occasions. In contrast to the ample evidence of avian predator activity, we

observed mammal tracks near enclosures on only two occasions, and snake tracks on only one occasion.

Discussion

We found strong evidence of predation on lizards at White Sands. An average of 36% of lizards survived in the open enclosure treatments during 2011, and 61% survived in 2012. In contrast, 100% of lizards survived in the closed treatments, which were identical to open treatments with the exception that predators were excluded. Additionally, 53% of predator observations occurred at the enclosure with the lowest mean lizard survivorship, which further suggests that lizard mortality was caused by predation in our study.

Avian predators were most likely responsible for the observed mortality in open enclosures. Visually oriented avian predators such as *Geococcyx californianus* (the greater roadrunner) and *Lanius ludovicianus* (the loggerhead shrike) occur at White Sands (Kaufman 1973; Raitt and Pimm 1976; Reid and Fulbright 1981), typically hunt during *H. maculata's* activity period (Calder 1965; Craig 1978; Hager 2001), and could easily enter and exit the open enclosures. In addition, we frequently observed *L. ludovicianus* in close proximity to enclosures, as well as *G. californianus* tracks, *Corvus cryptoleucus* (Chihuahuan raven) tracks, and small anisodactyl tracks (likely belonging to *Mimus polyglottos* [the northern mockingbird]) within enclosures. In contrast, we observed mammal tracks (likely belonging to *Canis latrans* [coyotes] and *Vulpes marcrotis* [kit foxes]) and snake tracks (likely belonging to *Pituophis catenifer* [Gophersnakes]) near enclosures on only a handful of occasions. We did not find evidence of mammals or reptiles (besides *H. maculata)* within our

enclosures at any time throughout the study, indicating that they were not responsible for the majority of the predation we observed.

Although lizards experienced high rates of predation in enclosure trials, survivorship did not differ significantly with respect to paint color treatment. This result is somewhat perplexing, given that previous studies offer strong evidence that blanched color is adaptive in White Sands (Rosenblum et al. 2004; Rosenblum et al. 2007; Rosenblum et al. 2010; Rosenblum and Harmon 2011). In a system amenable to experimental manipulation where divergence is literally in black and white, why did we fail to detect selection on color? There are a number of challenges with measuring natural selection in the wild, some of which may have contributed to our inability to detect an effect of color on survivorship. Here we use our results as a case study to examine six major categories of challenges frequently encountered in field-based studies of natural selection, and show that even in seemingly simple systems selection is often complex and dynamic.

Statistical Power

One challenge with detecting selection in the wild is obtaining a sample size large enough to observe statistically significant results. While statistical power can be an issue in any empirical study, it is particularly problematic in studies of selection in the wild because the strength of selection on morphological traits in natural populations is often quite weak (Endler 1986; Hereford et al. 2004; Hoekstra et al. 2001; Kingsolver et al. 2001). It can therefore be difficult (or even unethical) to attain the sample size necessary to detect the ongoing effects of selection, and consequently many previous studies have focused on species/populations where it is feasible to take a large number of individuals (e.g., Bolnick

2004; Diabaté et al. 2008; Funk 1998; Kaufman 1974; Vamosi and Schluter 2002; Via et al. 2000). To determine whether statistical power could have contributed to the absence of an effect of paint treatment on survival in our enclosure trials, we conducted a power analysis using the effect size of color on survivorship observed throughout the duration of the study. The power analysis indicated that a sample size of greater than 5000 lizards would be required to detect a significant difference in survivorship between substrate-matched and substrate-mismatched lizards. Obtaining such a large sample over the timeframe necessary to carry out the study would not only be unethical, but likely impossible, due to the small size and isolated nature of the White Sands population.

Sex and Life Stage Variations in Selection

A second category of challenges concerns selection that varies within a species with respect to sex and/or life stage of individuals. Previous studies indicate that the magnitude and direction of selection can vary between sexes and among life stages, with certain traits favored early in life but not later, or in one sex but not the other (e.g., Barrett et al. 2008; Forsman and Appelqvist 1999). This can have a huge impact on the outcome of studies of local adaptation, where depending on which groups researchers choose to focus on, observed patterns of natural selection could be completely different. In our study, we measured survivorship of adult males and females in enclosures. We found a marginally significant interaction between sex and paint color where substrate matching may have been more important for male survival than female survival. Specifically, males exhibited the expected pattern of higher survivorship of substrate-matched individuals than substrate-mismatched individuals, while in females the opposite was true. The most likely explanation for the

interaction effect is that there is a stronger relationship between substrate matching and survival for males than females. Male iguanid lizards are territorial and spend more time during the breeding season being behaviorally conspicuous with territorial and courtship displays (Martins 1994), and some prior research suggests that conspicuousness can have higher fitness costs for males than females in reptiles (Forsman and Shine 2008).

While we might have avoided the potentially confounding effect of variation between the sexes by focusing exclusively on selection in one sex, we did not originally anticipate that color would impact survival of males and females differently. In addition, based on power analyses using the magnitude of the effect of color on survivorship observed in males only, a sample size of approximately 350 males would be required to have a 95% chance of detecting a significant difference (nearly three times the number of males used in our study). We did not repeat the experiment with only males due to the concern that taking 350 males of reproductive age could reduce population size and genetic variation. We cannot evaluate the effect of life stage on survivorship in our study, because we focused exclusively on adults. However, if selection for substrate matching is stronger for juveniles than adults in White Sands, a possible explanation for the absence of a paint treatment effect is that we studied the "wrong" life stage.

Spatially and Temporally Variable Selection

Yet another challenge with measuring natural selection in the wild is the potential for spatial and temporal variation in selection. While most previous studies do not include spatial or temporal replicates (Kingsolver et al. 2001; Siepielsky et al. 2009), some studies have demonstrated that the magnitude and direction of selection on phenotypes can fluctuate over

space (i.e., geographic location) and/or time (e.g., year or season) (e.g., Caruso et al. 2003; Dobzhansky and Levene 1948; Gilbert et al. 1996; Gross et al. 1998; Maad 2000; Novembre and Di Rienzo 2009; Reimchen and Nosil 2002; Schemske and Horvitz 1989; Siepielsky et al. 2009; Totland 2001). Measuring aspects of fitness over too narrow a spatial or temporal scale can result in an incomplete picture of the impact of natural selection on ecologically relevant phenotypes.

Survivorship in our study varied dramatically with respect to space and time. Year, geographic location, and the interaction between year and geographic location had significant effects on individual survival. It is interesting to note that variation in survivorship occurred over extremely short spatial scales in our study, with significant differences between enclosures separated by a distance of only 110 meters. In addition, survivorship varied drastically from year to year at specific enclosures, with enclosures that exhibited high survivorship in 2011 exhibiting low survivorship in 2012 and vice versa. These patterns of variation could have to do with any number of aspects of the system's ecology, including biotic and abiotic factors such as predator activity and weather conditions (e.g., Grant and Grant 1993; Reznick 1982). For example, predator densities could fluctuate over small spatial scales depending on whether enclosures were located near suitable perches/nesting sites, and predator behavior could fluctuate over small temporal scales depending on whether trials took place during parts of the season where predators were defending territories, breeding, feeding chicks, etc. To investigate the effect of temporal and spatial variation on our ability to detect significant differences in survivorship, we conducted power analyses using the effect size observed within a single enclosure replicate where the magnitude of the effect of color was the strongest. A total of 84 lizards would be required to have a 95%

chance of detecting a significant difference, if selection over time and space had remained consistently strong (a sample one third the size of that used in our study). In other words, we would likely have detected an effect of paint treatment if variation between years and among sites had been lower, indicating that spatial and temporal variability had a considerable impact on our study.

Historical Versus Contemporary Selection

Studies of natural selection in the wild can be complicated by differences between historical and contemporary selection pressures. In addition to variation over small spatial and temporal scales (addressed above), selection pressures may also change over longer time periods, where the magnitude, direction, and/or mode of contemporary selection could be different than past dynamics. Historical selection pressures can have lasting effects on a population. For example, traits that originate in a population to allow individuals to avoid predation can remain widespread even if the dynamics of predation change such that the traits are no longer necessary for survival - a phenomenon termed “the ghost of predation past” (sensu Connell 1980). "Selection past" could in part explain our inability to detect an effect of color on survivorship in White Sands *H. maculata*. It is possible that color was historically more important for survivorship in White Sands, but contemporary selection is less intense due to changes in the ecology of the system. Changes in the abundance of predators of *H. maculata* have occurred in New Mexico in the recent past due to anthropogenic factors. For instance, *L. ludovicianus* has experienced declines across the country since 1966, with some of the highest negative trends occurring in regions of New Mexico (Cade and Woods 1997; Sauer 1995). If densities of visually oriented predators are

drastically different now than they were 50 years ago, we might not detect current differences in survival based on color even though there was historically strong selection for substrate matching. Selection on - or learning by - predators can also alter dynamics of selection on prey species, and could, in theory, result in predators adapted to detect blanched lizards on White Sands. However, the ecotone is narrow relative to the home range size of key avian predators in the region (e.g., *G. californianus* [Kelley et al. 2011]), making it likely that these predators regularly hunt in White Sands, ecotone, and dark soils habitats. We would therefore not expect that habitat-specific specialization in predators has shifted the dynamics of selection on White Sands lizards over time.

Selection on Correlated Traits

Selection on correlated traits is an additional factor that can complicate studies of natural selection in the wild. When the phenotypic values of multiple traits are correlated as a result of genetic covariances, selection on one trait can have indirect effects on correlated characters (Lande and Arnold 1983). Previous studies have found that correlation between traits can complicate measurement of phenotypic selection, making it difficult to determine whether traits that vary between populations are the direct targets of divergent selection or merely correlated with them (Arnold 1986; Clarke 1975; Endler 1986).

In White Sands *H. maculata*, body color is likely important for more than just crypsis; reptiles are ectothermic, and coloration affects an individual's ability to thermoregulate (Clusella et al. 2007). However, patterns of body color evolution in White Sands lizards appear to be in the opposite direction of what would be expected if thermoregulatory ability is the primary target of selection. Due to the unique thermal properties of gypsum, the

surface temperature of White Sands is much cooler than that of the surrounding desert. Previous research has shown that White Sands *H. maculata* captured in the field exhibit lower body temperature than dark soils individuals (Hager 2000), and our data demonstrate that painted lizards from matched and mismatched treatments exhibit similar thermoregulatory capabilities on White Sands substrate. It therefore seems unlikely that blanched color confers a fitness advantage to White Sands lizards in terms of thermoregulation- and in fact the opposite may be true. Blanched coloration could potentially make it more difficult for White Sands lizards to achieve and maintain preferred body temperature in the comparatively cool White Sands environment. In addition, the subset of animal species inhabiting White Sands that exhibit blanched coloration represent a variety of divergent taxa (including reptiles, amphibians, mammals, and invertebrates), and certainly do not all possess the same thermoregulatory mechanisms as *H. maculata*. Thus the convergence in color that has occurred among White Sands fauna is likely best explained by the shared necessity of avoiding predation, as opposed to thermoregulatory requirements. The effect of body color evolution on thermoregulation is an important area of future study in the White Sands system.

Deviations from Natural Conditions

A final challenge in experimental studies of selection is the difficulty of replicating natural conditions in an experimental context. Enclosure experiments often expose individuals to conditions that would not be encountered in nature, and these artificial conditions can have unanticipated effects that make it difficult to accurately measure selection (Endler 1986). For example, in our study each enclosure started at a density of 14

lizards per 100 square meters, which is a much higher than the density at which *H. maculata* naturally occur in White Sands (less than one lizard per 100 square meters [Hager 2001]). The density of conspecifics can affect predation rates experienced by a population (Lotka 1920; Volterra 1926). It is thus possible that we observed a "buffet effect" in our study, where visually oriented avian predators were initially attracted to the enclosures by substrate-mismatched lizards, but subsequently proceeded to consume lizards of both paint treatments. There was also a limited amount of available ground cover that all lizards within a given enclosure were required to share when seeking shelter from potential predators. It is possible that substrate matching alone is insufficient to avoid predation when escape opportunities are limited, and the abnormally high density of lizards within enclosures could have amplified this effect. The high density of lizards in our enclosures could also have caused undetected changes in behavior associated with increased foraging and competition for limited resources. Similarly, the paint treatment could have caused undetected changes in thermoregulatory behavior of lizards in the enclosures (although we did not observe differences in body temperature or thermal preference in the lab). Thus it is possible that the experimental design had unanticipated effects on other aspects of conspicuousness besides crypsis.

Conclusion

The challenges listed above are quite common - and we provided evidence that many may have affected our study. Our experiences, along with previously published research, indicate that decisions about the scope of a selection experiment can be particularly influential. For instance, choosing to exclude particular groups (e.g., focusing on only one

sex) can result in a misunderstanding of the dynamics of selection. In addition, choice of spatial/temporal scale (e.g., conducting a study over one or multiple years/geographic locations) can lead researchers to completely different conclusions about the direction and magnitude of selection on specific traits. Investigating the effects of "hidden" factors such as sex, life stage, time, and space almost always leads to a more accurate and nuanced picture of selection in wild. Preliminary work that can inform strategic decisions about the scope of an experimental study is particularly important.

Additionally, our study gives insight into the complications associated with experimentally manipulating vertebrates to learn about natural selection in the wild. In particular, experimental manipulation likely had a number of unintended consequences with respect to the behavior of our study organisms including lizard foraging behavior, escape behavior, thermoregulatory behavior, and social interactions. Experimental design could also have affected predator behavior (e.g., if the high density of prey within enclosures led predators to employ different hunting strategies). Experimental manipulation is a key tool for identifying phenotypic targets of natural selection. Complicating factors that arise as a byproduct of manipulating the study organism must be addressed through careful experimental design and data interpretation.

The results of our study indicate that, despite the difficulties detailed above, researchers often learn fascinating things about selection when experiments have unexpected outcomes. For instance, we documented spatial variation in predation over extremely fine scales in the White Sands system. Thus the White Sands system presents an exciting opportunity for future research to investigate scale-specific questions of how and why predation (and potentially selection) varies over space and time. In addition our data indicate

that an individual's sex likely plays a role in determining the effect of body color on survivorship, and thus future research at White Sands will focus on gaining an in-depth understanding of the causes of sex-specific differences in susceptibility to predation. The challenges encountered by our study - and the unexpected results revealed - represent exciting avenues for future research. As evolutionary biologists endeavor to better understand the adaptive significance of specific traits, it is important to consider factors that can generate complex patterns of natural selection even in seemingly simple systems. Studies that assess the effects of these factors on fitness are essential in gaining a nuanced, comprehensive, and accurate understanding of adaptation in the wild.

Figures

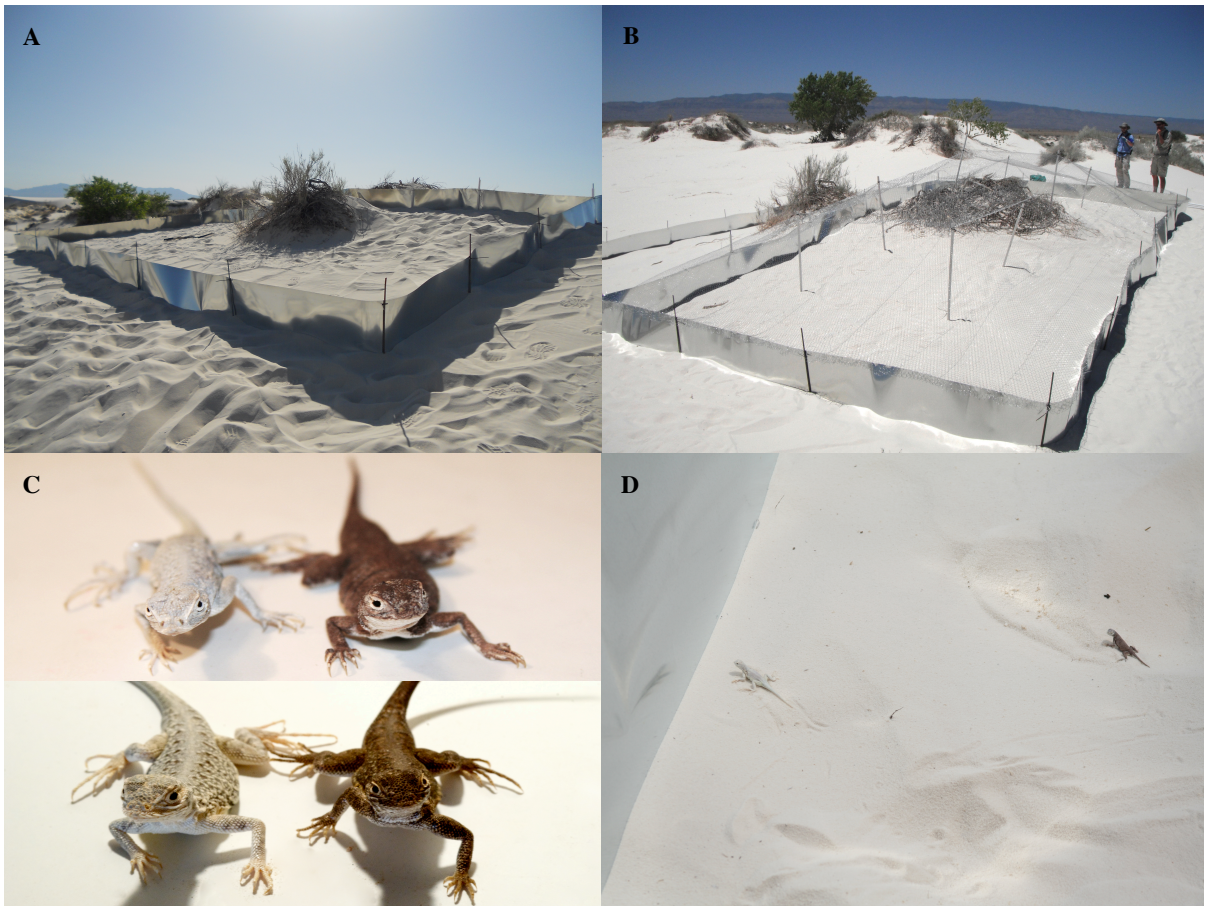

Figure 3.1: Design of the enclosure experiment. Panel A shows the "open" enclosure treatment, which allowed avian predators to enter and exit freely, and panel B shows the "closed" enclosure treatment, which excluded avian predators with chicken wire. The top half of panel C shows color-manipulated *H. maculata* (with the substrate-matched paint treatment on the left and substrate-mismatched paint treatment on the right), and the bottom half shows the corresponding naturally occurring color phenotypes (with the White Sands phenotype on the left and dark soils phenotype on the right) (photograph courtesy of S. Des Roches). Panel D shows a substrate-matched lizard (left) and a substrate-mismatched lizard (right) after release into an enclosure.

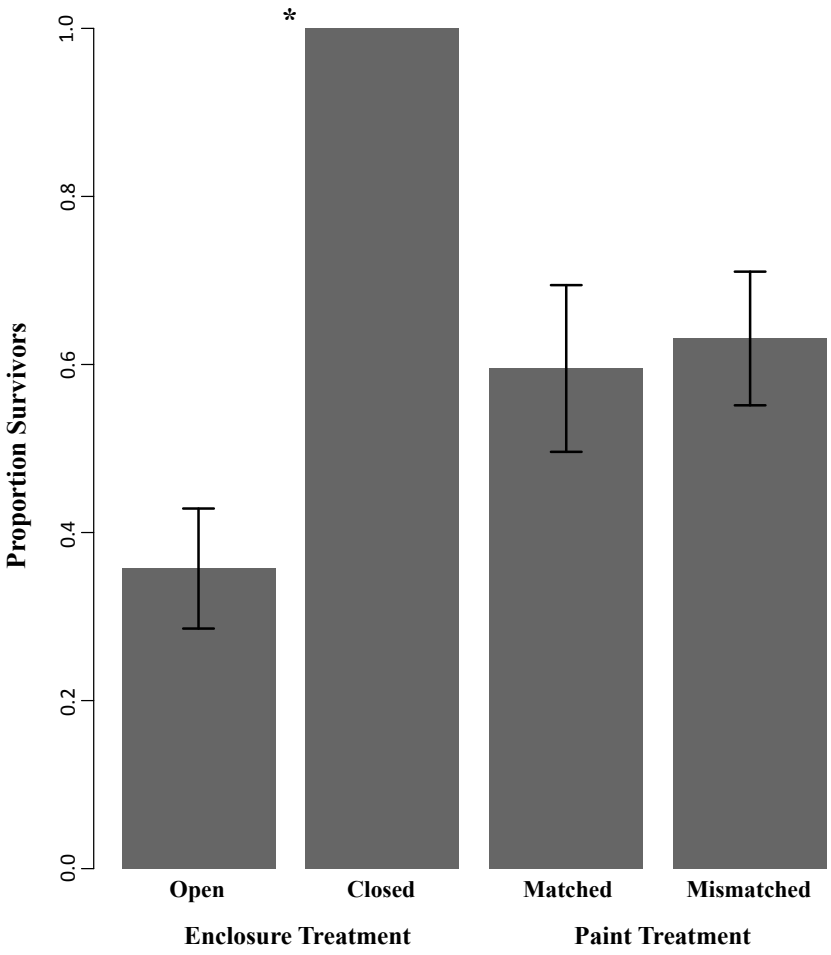

Figure 3.2: Proportion of lizards that survived trials with respect to enclosure treatment and paint treatment. Survivorship was significantly lower in the open enclosure treatment than in the closed enclosure treatment ($P < 0.05$ indicated by an "*"). We did not detect a significant difference in survivorship by paint treatment group. Bars represent mean survivorship across enclosure replicates, and vertical lines indicate the standard error of the mean. The open versus closed enclosure treatment comparison includes data from 2011 trials only (three open and three closed replicates, n = 84 lizards). The matched versus mismatched paint treatment comparison includes data from 2011 and 2012 trials (15 open replicates, n = 210 lizards).

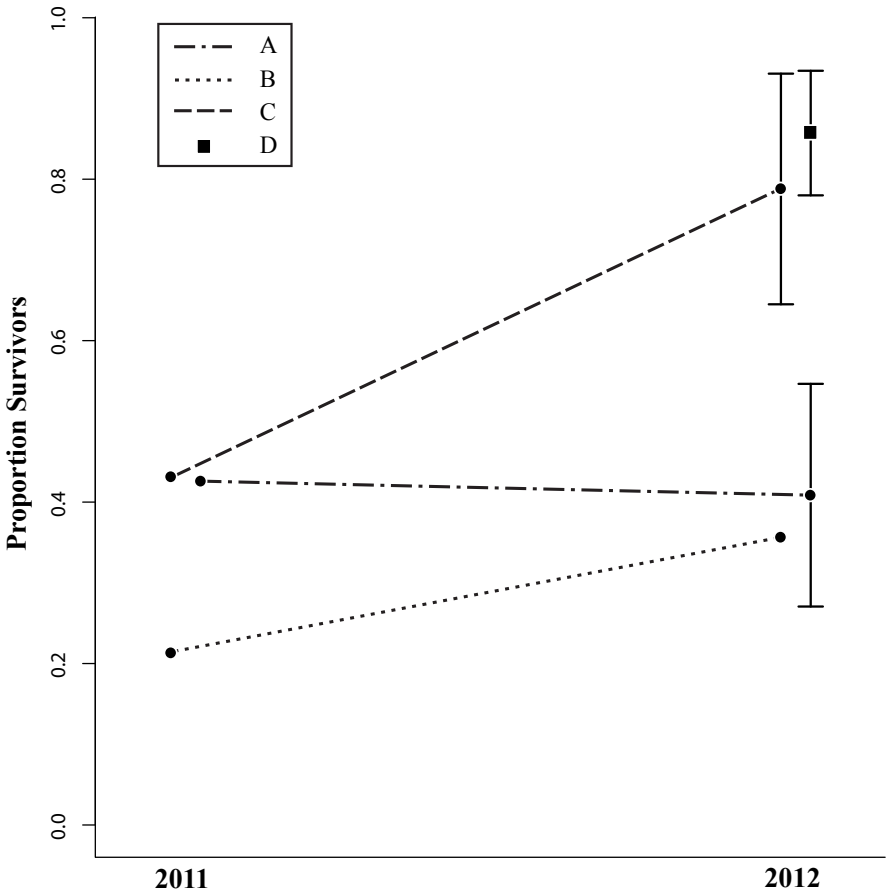

Figure 3.3: Proportion of lizards that survived trials with respect to year and enclosure location. Survivorship differed significantly between years ($P = 0.03$) and among enclosures ($P < 0.01$) in our general linear model. In addition, we detected an interaction between year and enclosure ($P = 0.03$), where survivorship in some enclosures differed significantly between years and survivorship in other enclosures did not. Dashed lines represent the interaction effect between year and enclosure on survivorship that we detected in our linear model, with endpoints representing mean proportion of survivors in replicates within different enclosures in different years. Solid, vertical lines indicate the standard error of the mean for enclosures in years with multiple replicates, where survivorship varied among replicates. We used open replicate data from each year to calculate survivorship for each enclosure (one replicate in 2011 and four in 2012 for enclosure A [n = 70 lizards]; one in 2011 and two in 2012 for enclosures B and C [n = 42 lizards each]; zero in 2011 and four in 2012 for enclosure D [n = 56 lizards]).

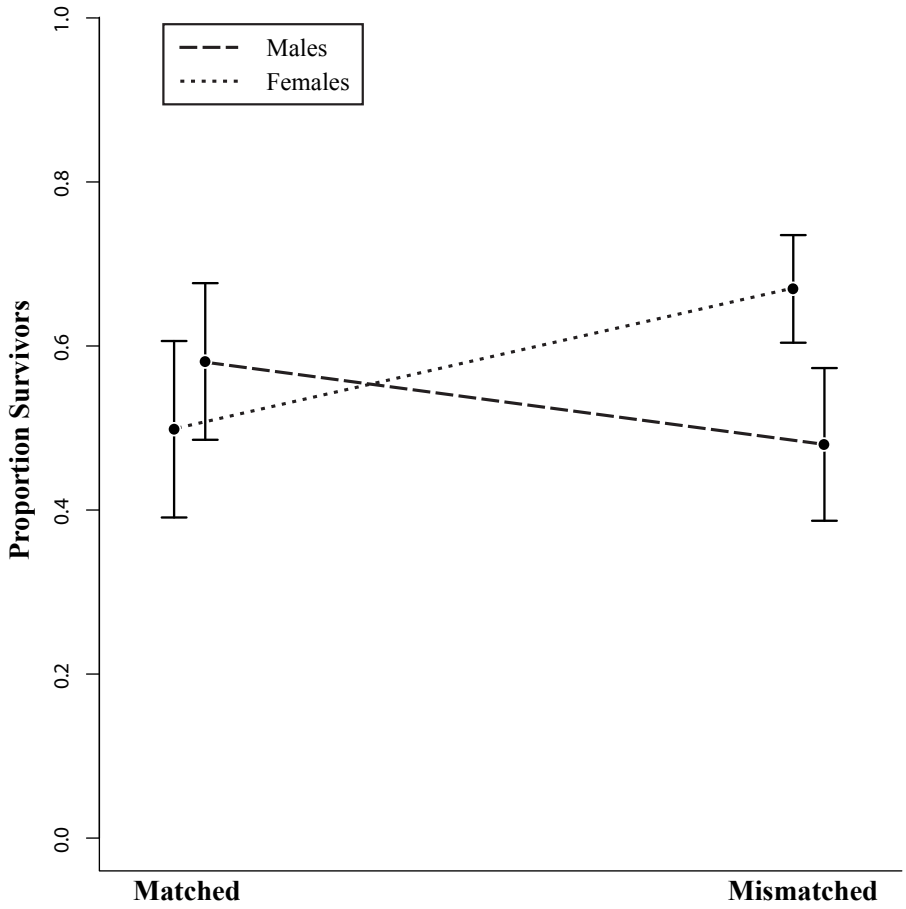

Figure 3.4: Proportion of lizards that survived trials with respect to paint treatment group and sex. We detected a marginally significant trend between sex and paint treatment in our general linear model (P = 0.05), where substrate-matched males had higher survivorship than substrate-mismatched males, but substrate-matched females had a lower survivorship than substrate-mismatched females. Dashed lines represent the interaction effect between sex and paint treatment on survivorship that we detected in our linear model, with endpoints representing mean proportion of survivors of each sex in different paint treatments across enclosure replicates. Solid, vertical lines indicate the standard error of the mean. We used open replicate data from 2011 and 2012 to calculate survivorship for each category (for a total of 15 open replicates, n = 210 lizards).

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CHAPTER IV

Quantitative Genetic Simulations Show That Standing Variation Facilitates the Evolution of Reproductive Isolation During Ecological Speciation

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Abstract

Ecological speciation is driven by adaptation, where divergent selection in distinct environments causes the evolution of reproductive isolation. Contemporary evidence suggests that standing genetic variation, ubiquitous within naturally occurring populations, is often responsible for seeding adaptation in the wild. Although researchers recognize the significance of standing variation in facilitating adaptation in nature, the impact of standing variation on the evolution of reproductive isolation during ecological speciation remains unexplored. To investigate the role of standing variation in ecological speciation we conducted computer simulations of populations undergoing adaptive divergence, where adaptation to distinct environments proceeded via either standing variation or new mutation. We quantified the accumulation of three types of reproductive isolation throughout simulations: behavioral isolation, extrinsic postzygotic isolation, and intrinsic postzygotic isolation. We found that populations accumulated reproductive isolation more rapidly and achieved higher overall levels of isolation when adaptation occurred from standing variation in some scenarios, depending on the level of migration between populations, time since divergence, the reproductive isolating mechanisms included in simulations, and the degree of mutational covariance between traits under selection. Our results indicate that standing

variation has the potential to play a key role in determining the outcome of the speciation process in real-world instances of ecological divergence.

Introduction

Ecological speciation is rapidly becoming accepted as an important mechanism for generating diversity in the natural world. Adaptation is a key component of the ecological speciation process, as divergent natural selection in distinct environments drives the evolution of reproductive isolation between populations (Schluter 1996). While initial population genetic models of adaptation (reviewed in Orr 2005a, 2005b) typically assumed the alleles that facilitate adaptation to novel selection pressures arise via new mutation, contemporary evidence suggests that the standing genetic variation within populations is often responsible for seeding adaptation in the wild (Barrett and Schluter 2007). Empirical studies have demonstrated that naturally occurring populations often harbor a high degree of genetic variation for ecologically relevant traits, and there are numerous documented examples of adaptation from standing variation in natural systems (e.g., Colosimo et al. 2005; Feder et al. 2003; Hoekstra et al. 2006). In addition, previous research on the genetic basis of adaptation suggests that the process of adaptation differs considerably when beneficial alleles are present in standing genetic variation versus when they arise via new mutation (reviewed in Barrett and Schluter 2007). Though researchers now recognize the importance of standing genetic variation in facilitating adaptation in the wild, models of ecological speciation still tend to focus on new mutation as the source of beneficial alleles, and thus the impact of standing variation on the evolution of reproductive isolation remains unexplored (Nosil 2012).

Although little work has been done to specifically investigate the role of standing variation in speciation, previous research suggests that standing variation may be important for the evolution of a variety of reproductive isolating mechanisms, including behavioral isolation, extrinsic postzygotic isolation, and intrinsic postzygotic isolation. Behavioral isolation occurs when individuals within diverging populations prefer local mates (Felsenstein 1981; Maynard Smith 1966). Previous empirical research has documented standing variation in traits important for assortative mating during ecological speciation (e.g., Seehausen et al. 2008), in addition to standing variation in alleles governing preference itself (reviewed in Bolnick and Fitzpatrick 2007). Extrinsic isolation occurs when hybrids between diverging populations express a phenotype that is intermediate between phenotypic optima (Rice and Hostert 1993). Extrinsic isolation is unique to ecological speciation in that it is driven by divergent adaptation (Schluter and Conte 2009), and thus standing genetic variation contributes to extrinsic isolation in any instances where it seeds adaptation. Finally, standing genetic variation has been documented in natural populations at loci involved in Bateson Dobzhansky Muller incompatibilities (BDMIs) (e.g., Sweigart et al. 2007), which are a form of intrinsic postzygotic isolation that occur when alleles existing separately in divergent populations have deleterious fitness effects when present together in hybrids (Bateson 1909; Dobzhansky 1934; Muller 1939, 1940, 1942).

There are a number of fundamental differences between adaptation from standing variation and adaptation from new mutation (e.g., Hermisson and Pennings 2005; Orr and Bettancourt 2001), all of which are almost certain to profoundly affect the dynamics of ecological speciation. Perhaps most relevant for speciation, adaptation to novel selection pressures is expected to be more rapid from standing variation than from new mutation.

When alleles that facilitate adaptation are present in standing variation, there is no waiting time for beneficial mutations to occur, meaning that adaptation can begin almost immediately (Barrett and Schluter 2007). In addition, beneficial alleles necessary for adaptation are present at a higher initial frequency in the standing variation scenario, and are thus less likely to be lost by drift than in the new mutation scenario (Hermisson and Pennings 2005). These findings indicate that ecological speciation should occur more quickly during adaptation from standing genetic variation compared with new mutation in certain scenarios, because factors that increase the speed with which adaptation occurs should in turn facilitate rapid ecological divergence and accelerate the evolution of reproductive isolation between populations.

In light of previous research demonstrating that standing genetic variation is common in natural populations and plays a central role in shaping the dynamics of adaptation, we sought to understand how standing variation affects the outcome of the ecological speciation process. We conducted computer simulations of populations undergoing ecological divergence, where divergent adaptation proceeded via either standing variation or new mutation. In our simulations, isolation between populations could evolve as a result of three possible mechanisms: behavioral isolation, extrinsic postzygotic isolation, and intrinsic postzygotic isolation. We quantified the accumulation of isolation over time in both standing variation and new mutation treatments to determine both how quickly isolation evolved in each treatment, and how much total isolation was achieved by each treatment at the end of simulations. We detected important differences between standing variation and new mutation treatments under some conditions, and show that reproductive isolation can evolve more rapidly and achieve higher overall levels of isolation under standing variation than when

adaptation occurs via new mutations. Differences between treatments depended on the level of migration between populations, time since divergence, the reproductive isolating mechanisms included in simulations, and the degree of mutational covariance between traits under selection. Our results suggest that it is important to consider standing variation in the context of other interacting factors in order to gain a complete understanding of the dynamics of ecological speciation, and we identify a number of specific scenarios in which standing variation has the potential to play an important role the speciation process in the natural world.

Materials and Methods

We simulated populations of individuals using code written in C++. We designed our simulations to represent adaptive divergence between two populations of a diploid, sexually reproducing animal species, and focused on conditions where adaptation to divergent environments occurred via either standing genetic variation or new mutation. Ultimately, our simulations included 24 scenarios representing different combinations of the following factors (described in Table 4.1): migration, with two possible categories depending on whether migration was allowed to occur between populations; reproductive isolating mechanisms, with four possible categories depending on whether mate preference and BDMIs were included; and degree of mutational covariance, with three possible categories depending on whether there was positive, negative, or zero covariance between traits. We ran 20 replicates of all 24 simulation scenarios for both the standing variation and new mutation treatments. Below we describe the specifics of our simulations program, along with the parameter values used to generate each simulation scenario.

Simulations

We conducted individual-based simulations. Each individual in our populations was haploid with two traits determined by the additive effects of 50 fully pleiotropic unlinked loci. In our program, each generation of a simulation included three life cycle stages: selection, reproduction, and migration. During the selection stage, each population experienced stabilizing selection toward a particular phenotypic optimum. We determined an individual's extrinsic fitness according to the following equation:

$$W(z) = e^{-\gamma(z-\Theta)^2} \quad \textbf{(1)}$$

where $W(z)$ is the individual's fitness based on its multivariate phenotype, z is a two-element vector describing an individual's trait values, Θ is a two-element vector describing the location of the phenotypic optimum, and γ is a matrix describing the strength of stabilizing selection (Lande 1979). The diagonal elements of γ describe the strength of selection acting directly on each trait, whereas the off-diagonal elements invoke correlational selection. We did not include correlational selection in our simulations, and thus the off-diagonal elements of γ were always equal to zero. During selection an individual's probability of survival was equal to $W(z)$. We imposed hard selection in our simulations, meaning that survival of an individual was determined by the individual's fitness alone, rather than by the fitness of the individual relative to the fitness of others in the population.

During the reproduction stage, we generated offspring within each existing population. In our simulations mate preference (described below) determined whether mating occurred between particular individuals. To produce each new offspring, we drew pairs of

putative parents at random from the population until we found two individuals that would mate. The offspring received an allele from one of the two parents at each locus (with free recombination between loci). There was a 0.000001 probability of mutation at each locus. When a mutation occurred, we drew a mutational effect from a multivariate normal distribution and added it to the current value at that locus to generate a new allele. The mean of the multivariate distribution was zero, and the variance for both traits was 0.05. The covariance of the multivariate distribution depended on the simulation scenario (Table 4.1). The magnitude of the correlation between mutations in traits one and two, r_{μ}, was 0.5 in the positive covariance scenario, -0.5 in the negative covariance scenario, and zero in the no covariance scenario. Environmental variance in phenotype was equal to zero. We generated offspring in the above-described manner until the number of new individuals in each population was equal to its designated population size, which depended on the simulation phase (see below). No parents survived into the next generation.

Finally, migration occurred. Each individual migrated between populations with a probability *m*. The parameter *m* varied depending on the simulation scenario, with values of 0.01 for scenarios with migration, and values of zero for scenarios without migration (Table 4.1). We chose the final destination of each migrant individual randomly from all existing populations, meaning each migrant had an equal chance of either moving to a new population or remaining within its original population.

We designed separate treatments to simulate adaptation from standing genetic variation and adaptation from new mutation. In the standing genetic variation treatment, simulations included two phases: a phase that allowed the accumulation of genetic variation, and a phase where populations were subjected to divergent selection. We initiated the

variation accumulation phase with one population of 10,000 individuals. Individuals were initially genotypically and phenotypically monomorphic, with an allele at each locus that had an additive effect of zero on the phenotype. To allow the accumulation of genetic variation, we subjected the population to weak stabilizing selection (diagonal elements of $\gamma = 0.0001$) with an optimum trait value at the origin [0, 0] for 200,000 generations. We chose the number of individuals, number of loci, and number of generations to allow populations to build up sufficiently high levels of standing variation and achieve mutation-selection balance under weak stabilizing selection with the specified mutation parameters (Burger and Lande 1994). We then randomly split the large population into two separate, smaller populations of 5,000 individuals each. We subjected these populations to strong divergent selection (diagonal elements of $\gamma = 0.01$) toward distinct phenotypic optima (located at [5,5] and [-5,-5]) for 10,000 generations, which was long enough for high levels of reproductive isolation to evolve between populations in most scenarios. In contrast, in the new mutation treatment, we initialized two populations of 5,000 monomorphic individuals. To simulate adaptation from new mutation, these populations entered the divergent selection phase without being allowed to accumulate genetic variation. They were thus immediately subjected to strong divergent selection (diagonal elements of $\gamma = 0.01$) toward distinct phenotypic optima (located at [5,5] and [-5,-5]) for 10,000 generations.

We included mechanisms for both pre- and postzygotic reproductive isolation to evolve in our simulations. To facilitate the evolution of prezygotic behavioral isolation, individuals in some simulation scenarios exhibited mate preference (Table 4.1). Preference in our simulations followed a one-allele mechanism of assortative mating (Felsenstein 1981), where individuals preferred to mate with others with similar phenotypic values for traits

under divergent selection. In simulations with mate preference, the probability that mating occurred between two randomly chosen individuals was described by the following equation:

$$P(Mate) = 0.5\left(e^{-\alpha(z_{1,1}-z_{2,1})^2}\right) + 0.5\left(e^{-\alpha(z_{1,2}-z_{2,2})^2}\right) \quad \textbf{(2)}$$

In this equation, $z_{1,1}$ and $z_{2,1}$ represent values of trait one for individuals one and two, respectively; $z_{2,1}$ and $z_{2,2}$ represent values of trait two for individuals one and two, respectively; and α describes the intensity of assortative mating. In our simulations α was equal to 0.1 in replicates with preference, and zero in replicates with no preference.

To facilitate the evolution of postzygotic intrinsic isolation, we allowed BDMIs to accumulate in some simulations (Table 4.1). In simulations with BDMIs, each individual had an additional 512 BDMI loci. These BDMI loci were unlinked with one another and with the 50 trait-coding loci, and did not have an effect on phenotypic traits. In addition, the 512 BDMI loci in population one did not correspond to those in population two; i.e., mutations that occurred in the two populations occurred at different loci. This is because we are using these loci to capture the effect of new mutations that arise in each population that might be incompatible with new mutations that arise in the other population; given the large size of the genome, we consider the chances of mutations at the same locus in both populations to be small enough to ignore. We initiated individuals with values of [0] at all BDMI loci, and individuals inherited alleles at BDMI loci following the same methods as at trait-coding loci. During reproduction, alleles mutated from the ancestral state [0] to the derived state [1] with a per-locus probability of $\mu_{BDMI} = 0.0003$ (we did not allow mutations from the derived to the ancestral state). When a mutation occurred at a particular locus for the first time in the

history of a population, the derived allele at that locus had a chance of causing an incompatibility with a derived allele in the opposite population. We calculated the probability of incompatibility as 0.0075 (the probability that a given pair of alleles were incompatible) multiplied by the number of derived alleles segregating in opposite population. If an incompatibility occurred, we chose the incompatible locus randomly from the set of loci with derived alleles segregating in the opposite population. If mating occurred between individuals carrying incompatible alleles, the resulting offspring suffered a negative fitness effect. Specifically, each pair of incompatible alleles (i.e., each incompatibility) multiplicatively reduced the fitness of an individual by 25%. In other words, to find individual fitness in simulations with BDMIs, we first calculated the fitness effect of BDMIs as 0.75 raised to the power of the number of incompatibilities an individual carried. We then multiplied the BDMI fitness effect by the extrinsic fitness of the individual to determine overall fitness, and used this value to determine whether the individual survived selection according to the same methods as those employed in simulations without BDMIs.

We ran 20 replicates of each simulation scenario for both the new mutation and standing variation treatments, collecting genetic and phenotypic data from populations every 100 generations during the divergent selection phase. In standing variation simulations, there was no migration, mate preference, or mutation at BDMI loci during the variation accumulation phase.

Phenotypic and Genetic Divergence

We quantified divergence between populations throughout our simulations. To measure phenotypic divergence we calculated the mean and variance in phenotypic traits for

each population at each time point during the divergent selection phase of simulations. To measure genetic divergence between populations we calculated the fixation index at each time point using the formula $F_{ST} = 1 - {H_S}/{H_T}$. To find H_S we calculated heterozygosities at each locus using allele frequencies from each population separately, then averaged heterozygosity values across loci and between populations. To find H_T we calculated heterozygosities at each locus using averages of allele frequencies between populations, then averaged heterozygosity values across loci. We calculated locus-specific heterozygosity values as $H = 1 - \sum_{i=1}^{n} x_i^2$, where n is the number of alleles segregating at that locus and x_i is the frequency of the ith allele (Hartl and Clark 1997; Gillespie 2004).

Evaluating the Evolution of Reproductive Isolation

We measured the evolution of three kinds of reproductive isolation in simulations: prezygotic behavioral isolation, postzygotic extrinsic isolation, and postzygotic intrinsic isolation. We measured the contribution of each type of isolating mechanism to overall reproductive isolation following the methods first proposed by Coyne and Orr (1989), and expanded upon by Ramsey et al. (2003). To evaluate the evolution of behavioral isolation, we conducted mate preference trials every 100 generations throughout the simulations. Specifically, at each data collection time point, we randomly sampled 1,000 individuals from each population and placed them temporarily within a single test population. We then chose individuals randomly from within the test population and determined the probability that they would mate using equation (2). If the calculated probability was smaller than a random number drawn between zero and one, mating occurred. We continued this process until 1,000 matings had taken place, keeping track of how many of the matings were hybridizations

between individuals from different populations. Finally, we calculated behavioral reproductive isolation for that time point according to the following equation:

$$RI_{behavioral} = 1 - (\frac{number\ of\ hybridizations}{number\ of\ non\text{-}hybridizations}) \quad \textbf{(3)}$$

To evaluate the evolution of both intrinsic and extrinsic isolation, we compared fitness values between hybrid and non-hybrid offspring. At each data sampling time point, we generated 1,000 hybrid and 1,000 non-hybrid offspring. To generate each hybrid we randomly selected a parent individual from each divergent population and produced an offspring between them according to previously described methods for reproduction. We did not allow mate preference during offspring production, such that each pair of randomly drawn parents necessarily produced a hybrid. To produce non-hybrid offspring we followed the same procedure for producing hybrids, with the exception that pairs of parents were drawn from within the same population. We produced 500 non-hybrid offspring by mating pairs of individuals from population one, and 500 non-hybrid offspring by mating pairs of individuals from population two. We measured extrinsic fitness of both hybrid and non-hybrid offspring using equation (1). For each individual we calculated separate values of extrinsic fitness at each multivariate phenotypic optimum available in the divergent selection phase, and used whichever fitness value was highest in the calculation of extrinsic reproductive isolation. We calculated extrinsic reproductive isolation for a particular time point according to the following equation:

$$RI_{extrinsic} = 1 - (\frac{mean\ extrinsic\ fitness\ of\ hybrids}{mean\ extrinsic\ fitness\ of\ non{-}hybrids}) \quad \textbf{(4)}$$

We measured intrinsic fitness of hybrid and non-hybrid offspring by calculating the BDMI fitness effect based on the number of incompatibilities an individual carried, as described previously. We then determined intrinsic reproductive isolation for a particular time point according to the following equation:

$$RI_{intrinsic} = 1 - \left(\frac{mean\ intrinsic\ fitness\ of\ hybrids}{mean\ intrinsic\ fitness\ of\ non-hybrids}\right) \quad \textbf{(5)}$$

We calculated the absolute contributions of each reproductive isolating barrier to total reproductive isolation using the general formula:

$$AC_n = RI_n\left(1 - \sum_{i=1}^{n-1} AC_i\right) \quad \textbf{(6)}$$

More specifically, $AC_1 = RI_{behavioral}$, $AC_2 = RI_{intrinsic}(1 - AC_1)$, and $AC_3 = RI_{extrinsic}[1 - (AC_1 + AC_2)]$. We calculated total reproductive isolation as:

$$RI_{total} = AC_1 + AC_2 + AC_3 \quad \textbf{(7)}$$

Statistical Analysis

In order to determine whether standing genetic variation influenced the evolution of reproductive isolation between populations, we compared the amount of reproductive isolation that had evolved at different time points between the standing variation and new mutation treatments. Because simulation-based studies are typically not restricted in the number of replicates that they can generate, it is generally inappropriate to implement

statistical tests where the inference of significance is influenced by sample size. We therefore determined statistical significance at particular time points by calculating the difference in reproductive isolation values for randomly selected pairs of standing variation and new mutation replicates. Treatments were significantly different if at least 95% of difference values were greater than (or less than) zero. In order to avoid issues associated with multiple comparisons, we performed tests for statistical significance at a subset of the data-collection time points: 1,600 generations (at which point the magnitude of the difference between standing variation and new mutation treatments was greatest for most scenarios), 5,000 generations, and 10,000 generations. We will subsequently refer to these data-collection points as time points 1, 2, and 3. To further explore differences among simulation scenarios, we calculated a number of summary statistics. To compare patterns of accumulation of reproductive isolation over time between standing variation and new mutation treatments, we calculated median, minimum, and maximum reproductive isolation values among replicates at different time points. We also determined the proportion of replicates that had reached 50%, 75%, and 95% reproductive isolation at particular time points in different scenarios. Finally, as a measurement of the level of variability within standing variation and new mutation treatments, we calculated the difference between the maximum and minimum values of reproductive isolation observed among replicates at particular time points. We conducted all statistical analyses in R vers. 3.1.2 (R Development Core Team 2014).

Results

In our simulations, population divergence and the evolution of reproductive isolation generally occurred more rapidly for the standing variation treatment than the new mutation

treatment. The magnitude of the effect of standing variation depended on a number of factors including whether or not migration occurred between populations, the amount of time since population divergence, the reproductive isolating mechanisms included in simulations, and the degree of mutational covariance between traits. Below we detail differences between standing variation and new mutation treatments in the evolution of reproductive isolation over time, focusing initially on simulations that included migration, all possible isolating mechanisms, and no mutational covariance. We subsequently explore how isolation evolved over time in simulations with no migration, different combinations of isolating mechanisms, and positive/negative mutational covariance.

Migration Rate

Migration had a strong effect on the evolution of reproductive isolation in our simulations. In simulations that included migration (in addition to all possible isolating mechanisms and no mutational covariance), total reproductive isolation was higher in the standing variation treatment in 95% of pairwise comparisons between standing variation and new mutation replicates at generation time point 1 (Figure 4.1). In contrast, in the absence of migration we did not detect a significant difference between treatments (i.e., only 70% of pairwise comparisons were higher for standing variation replicates). Thus migration was key in generating differences in the evolution of reproductive isolation between the standing variation and new mutation treatments. In addition, simulations with migration generally attained high levels of reproductive isolation less rapidly than those without migration. For example, 100% of replicates had reached isolation values of greater than 0.95 by between time points 2 and 3 in simulations with migration, whereas in simulations without migration,

100% of replicates had reached isolation values of greater than 0.95 before time point 1 (Figure 4.1). Finally, migration tended to generate increased levels of variation among simulation replicates, with variation reaching a maximum of 1.02 in simulations with migration, compared with 0.48 in simulations without migration (Figure 4.1).

Time Since Divergence

As expected, populations diverged genetically and phenotypically over the course of the divergent selection phase of simulations in both standing variation and new mutation treatments. In simulations that included all possible isolating mechanisms, in addition to migration and no mutational covariance, the majority of replicates exhibited F_{ST} values of 0.75 or greater by time point 1, and values of 0.95 or greater by time point 2. Phenotypic divergence between populations followed a similar pattern, with difference in mean phenotype between populations increasing over time in both treatments.

Differences between standing variation and new mutation treatments in evolution of reproductive isolation fluctuated over time, with significant differences between treatments occurring early in simulations. Reproductive isolation was significantly higher in standing variation treatments at time point 1, with median reproductive isolation values of 0.84 (minimum = 0.13; maximum = 0.99) for the standing variation treatment and 0.23 (minimum = -0.02; maximum = 0.51) for the new mutation treatment (Figure 4.1). In addition, 75% of standing variation replicates had reached reproductive isolation values of at least 0.5 by time point 1, compared with just 5% of new mutation replicates.

In contrast, reproductive isolation values were not significantly different between standing variation and new mutation treatments midway through simulations, with standing

variation replicates exhibiting greater values than new mutation replicates in only 65% of pairwise comparisons at time point 2. Median values of reproductive isolation at time point 2 were > 0.99 (minimum = 0.86; maximum > 0.99) for the standing variation treatment, and > 0.99 (minimum = 0.52; maximum > 0.99) for the new mutation treatment (Figure 4.1). By time point 3, median, minimum, and maximum values for both standing variation and new mutation treatments were > 0.99 (Figure 4.1). Thus differences between standing variation and new mutation treatments in the evolution of reproductive isolation were typically most pronounced in the early stages of divergence between populations, and tended to diminish over time.

Combination of Reproductive Isolating Mechanisms

The magnitude of the difference between standing variation and new mutation treatments also depended on the combination of reproductive isolating mechanisms included in simulations. When we removed mate preference and BDMIs, standing variation and new mutation treatments were not significantly different early in simulations, with only 60% of pairwise comparisons of replicates greater for the standing variation treatment at time point 1 (Figure 4.2). Thus extrinsic isolation alone was not sufficient to generate differences in reproductive isolation between treatments. In addition, reproductive isolation values remained low over time for scenarios without preference and BDMIs, with median values of 0.10 (minimum = -0.03; maximum = 0.21) for standing variation and 0.08 (minimum = -0.04; maximum = 0.19) for new mutation at time point 1; 0.12 (minimum = 0.02; maximum = 0.21) for standing variation and 0.16 (minimum = 0.04; maximum = 0.27) for new mutation at time point 2; and 0.12 (minimum = -0.02; maximum = 0.22) for standing

variation and 0.13 (minimum = 0.05; maximum = 0.21) for new mutation at time point 3 (Figure 4.2).

We also observed an interaction effect between reproductive isolating mechanisms in our study. In simulations with mate preference and without BDMIs, the effects of individual isolating mechanisms on overall isolation were not simply additive; the degree of reproductive isolation we observed in standing variation simulations was greater when both mate preference and BDMIs were present than what we would expect given the effects of each mechanism separately (i.e., median reproductive isolation at time point 1 was 0.84 [minimum = 0.13; maximum = 0.99] when both mechanisms were present; 0.37 [minimum = 0.11; maximum = 0.87] with preference only; and 0.26 [minimum = 0.14; maximum = 0.51] with BDMIs only) (Figure 4.2). The opposite was true for new mutation simulations, with the degree of reproductive isolation when both mechanisms were present being lower than the sum of the effects of each mechanism separately (i.e., median reproductive isolation at time point 1 was 0.23 [minimum = -0.02; maximum = 0.51] when both mechanisms were present; 0.18 [minimum = -0.05; maximum = 0.47] with preference only; and 0.17 [minimum = 0.0005; maximum = 0.36] with BDMIs only) (Figure 4.2).

To further explore the observed interaction effect, we calculated the absolute contributions of mate preference and BDMIs to total reproductive isolation in trials with both isolating mechanisms, as well as in trials with just one mechanism or the other. We then determined the generation at which the difference between median absolute contribution values for standing variation and new mutation replicates was the greatest for each simulation scenario. With either isolating mechanism alone, we observed the greatest difference between absolute contribution values later in simulations (i.e., at generation 2,000

for trials with preference only, and generation 3,600 for trials with BDMIs only) than with both isolating mechanisms together (i.e., at generation 1,700). Thus interactions between multiple isolating barriers were important for facilitating rapid accumulation of reproductive isolation, as well as for generating differences between the standing variation and new mutation treatments.

Mutational Covariance

Finally, mutational covariance had a strong effect on the evolution reproductive isolation in our simulations. Patterns of evolution of reproductive isolation in simulations with all possible isolating mechanisms and positive mutational covariance were similar to those observed in trials with no mutational covariance; that is, 100% of pairwise comparisons of reproductive isolation were higher for standing variation replicates than new mutation replicates at time point 1, with differences between standing variation and new mutation diminishing over time (i.e., only 60% of comparisons were higher for standing variation at time point 2) (Figure 4.3). In addition, the effect of standing variation was of greater magnitude in trials with positive covariance than in trials with no covariance. For instance, the difference between median values of reproductive isolation for standing variation and new mutation treatments was the greatest before time point 1 for trials with positive covariance, while it was greatest between time points 1 and 2 for trials with no covariance (Figure 4.3). Thus positive mutational covariance tended to exaggerate differences between standing variation and new mutation treatments when both mate preference and BDMIs were present.

However, when we removed mate preference, we saw a different effect of positive covariance. Levels of reproductive isolation remained low throughout the divergent selection phase of no preference simulations, with observed median reproductive isolation values of 0.09 (minimum = 0.003; maximum = 0.91) for standing variation replicates and 0.06 (minimum = -0.04; maximum = 0.015) for new mutation replicates at time point 1; and 0.19 (minimum = 0.06; maximum > 0.99) for the standing variation treatment, and 0.20 (minimum = 0.11; maximum = 0.29) for the new mutation treatment at time point 3 (Figure 4.3). In addition, the standing variation treatment exhibited much higher levels of variation than the new mutation treatment in simulations with positive covariance. Variation in reproductive isolation among replicates peaked at 1.09 for the standing variation treatment, compared with just 0.37 for the new mutation treatment (Figure 4.3). Comparisons of genetic divergence shed further light on this pattern- F_{ST} values remained low throughout most of the positive covariance, BDMI-only replicates, with median values at time point 3 of 0.50 (minimum = 0.46; maximum = 0.97) for standing variation, and 0.51 (minimum = 0.48; maximum = 0.55) for new mutation. Thus positive mutational covariance suppressed genetic divergence and the evolution of reproductive isolation between populations when mate preference was absent.

The evolution of reproductive isolation also differed in the negative mutational covariance simulations compared with positive covariance and no covariance scenarios. In simulations with negative covariance and all possible isolating mechanisms, high levels of reproductive isolation evolved (median > 0.99 at time point 3 for both treatments), with no significant differences between standing variation and new mutation treatments in reproductive isolation time point 1 (Figure 4.4). In the negative covariance scenario when we

removed preference, we observed low levels of reproductive isolation similar to those in positive covariance, no-preference simulations, where median reproductive isolation at time point 3 was 0.07 (minimum = -0.06; maximum > 0.99) for standing variation and 0.06 (minimum = -0.09; maximum = 0.12) for new mutation (Figure 4.4).

Notably, in simulations with negative covariance and no BDMIs, we observed a substantial difference between standing variation and new mutation treatments, where the degree of reproductive isolation that evolved in standing variation simulations was much greater than in new mutation simulations. At time point 1, we observed median reproductive isolation values of 0.19 (minimum = -0.004; maximum = 0.56) for standing variation and 0.03 (minimum = -0.07; maximum = 0.13) for new mutation, with 90% of pairwise comparisons larger for the standing variation treatment (Figure 4.4). At time point 2, median values of reproductive isolation were 0.55 (minimum = 0.28; maximum = 0.83) for standing variation and 0.05 (minimum = -0.08; maximum = 0.18) for new mutation, with 100% of pairwise comparisons larger for the standing variation treatment (Figure 4.4). Finally, at time point 3, median values of reproductive isolation were 0.83 (minimum = 0.64; maximum = 0.92) for standing variation and 0.19 (minimum = 0.04; maximum = 0.37) for new mutation, with 100% of pairwise comparisons larger for the standing variation treatment (Figure 4.4). This represents the greatest difference between new mutation and standing variation treatments that we observed in our study. F_{ST} values remained low throughout most negative covariance, preference-only replicates in the new mutation treatment, with a median value at time point 3 of 0.48 (minimum = 0.33; maximum = 0.59); in contrast, median F_{ST} values increased over time in the standing variation treatment, with a median value at time point 3 of 0.95 (minimum = 0.88; maximum = 0.97). Thus in preference-only simulations, negative

mutational covariance amplified differences between standing variation and new mutation treatments in both genetic divergence and the evolution of reproductive isolation.

Discussion

Our results indicate that standing genetic variation can strongly affect the evolution of reproductive isolation during ecological divergence. Specifically, reproductive isolation accumulates more rapidly between populations when adaptation occurs from standing genetic variation as opposed to new mutation in many scenarios. Our results also indicate that the effect of standing variation on reproductive isolation interacts in complex ways with several factors including migration, time since divergence, reproductive isolating mechanisms, and mutational covariance. Below we discuss potential explanations for the importance of each of these factors in generating observed patterns of reproductive isolation in our study. We also address the significance of our results in terms of understanding the role of standing variation in speciation in the natural world.

Migration played a central role in determining the effect of standing variation on the evolution of reproductive isolation in our simulations. Specifically, in simulations without migration, we did not observe any significant differences in the accumulation of isolation between standing variation and new mutation treatments (Figure 4.1). This is likely because in the absence of the homogenizing effect of migration, populations in both treatments were able to respond to selection and adapt to distinct peaks extremely rapidly. In contrast, because gene flow can inhibit population response to selection (Haldane 1930; Mayr 1942; Nagylaki 1975; Slatkin 1987), factors that increased the effectiveness of isolating mechanisms at initiating divergence (such as standing variation) were of elevated importance

in simulations with migration. Our results suggest that standing variation is particularly important in facilitating the evolution of reproductive isolation during divergence with gene flow, and is perhaps less important in the context of allopatric speciation.

The accumulation of reproductive isolation in our simulations also varied over time. Specifically, in some categories, standing variation replicates exhibited higher amounts of isolation than new mutation replicates early in simulations, while in the late stages of simulations when populations were mostly reproductively isolated from one another the two treatments exhibited similar amounts of isolation (Figure 4.1). This is concordant with theory related to the genetics of adaptation, which predicts that adaptation should occur more rapidly from standing variation because beneficial alleles are already present in the population at the time the new environment becomes available (Barrett and Schluter 2007). The more rapidly populations adapt to divergent environments, the more rapidly they can become phenotypically and genetically differentiated and begin to accumulate reproductive isolation. Standing variation did not usually result in different outcomes to the speciation process in our simulations, with new mutation replicates eventually evolving similar amounts of reproductive isolation to standing variation replicates in most scenarios. However, the accelerated evolution of reproductive isolation that we observed early in simulations is likely to have consequences for the outcome of the speciation process in the real world. Mechanisms that facilitate the evolution of reproductive isolation are critical during the early stages of sympatric/parapatric speciation, where ongoing gene flow often strongly inhibits divergence (Kondrashov 1986). In addition, parameters in our simulations were static, but the real world is frequently variable (e.g., Gibbs and Grant 1987; Grant and Grant 1989; Grant and Grant 1993). Temporal fluctuations in factors such as strength of selection can inhibit

speciation (e.g., Bolnick 2011; Johansson and Ripa 2006), and thus the rapid accumulation of reproductive isolation in the early stages of divergence is often essential for the maintenance of population differentiation.

The evolution of reproductive isolation also varied based on which isolating mechanisms we included in simulations. Extrinsic ecological isolation, when acting alone, generated levels of reproductive isolation around 0.15 (Figure 4.2). This is expected given that individual extrinsic fitness depended on distance from divergent phenotypic optima, and hybrids between diverging populations typically occupied a position in phenotypic space intermediate to the two optima. Assuming a mean hybrid phenotype that is exactly intermediate to the two phenotypic optima, the maximum possible value of extrinsic reproductive isolation is equal to 0.22 given the selective surface in our simulations. If optima had been located farther apart in phenotypic space, extrinsic isolation could possibly have made a greater absolute contribution to total isolation. In addition, previous research has suggested that multifarious selection is more likely to lead to ecological speciation (e.g., Slatkin 1982; Doebeli and Dieckmann 2003; Gavrilets 2004). It is possible that increasing the dimensionality of selection could have increased the effect of standing variation on the evolution of extrinsic isolation in our simulations.

Mate preference and BDMIs, when acting individually, differed in the time points at which they most significantly affected reproductive isolation. Mate preference generated differences between standing variation and new mutation treatments early on in simulations. For example, in the preference-only scenario standing variation and new mutation simulations differed to the highest degree in the first 2,000 generations (Figure 4.2). This is likely because one-allele mechanisms of mate preference require some level of phenotypic

variation to initiate divergence (Kirkpatrick & Ravigne 2002). In fact, the one-allele mechanism of preference appeared to inhibit divergence early in simulations in the new mutation treatment as a result of the low initial levels of phenotypic variation within populations.

BDMIs generated differences between standing variation and new mutation treatments slightly later on in simulations. For example, in the BDMIs-only scenario, the difference between standing variation and new mutation simulations peaked at generation 3,600 (Figure 4.2). This is likely because some level of population divergence is required before incompatibilities can accumulate between populations (Orr 1995; Orr and Turelli 2001). Previous empirical studies have found higher degrees of intrinsic isolation between populations that are ecologically differentiated than those that are not (e.g., Funk et al. 2006; Bolnick et al. 2006), and our results suggest that standing variation could play a role in generating this pattern by facilitating adaptive divergence and allowing incompatibilities to accumulate.

We also observed an interaction effect between reproductive isolating mechanisms in our simulations, where isolation accumulated even more rapidly in standing variation replicates when both mate preference and BDMIs were present than when just one mechanism was present (Figure 4.2). This likely occurred because when both mechanisms are present, they are able to amplify the effects of one another. Mate preference initiates divergence, and BDMIs start to accumulate in response; the accumulated BDMIs further increases divergence, which in turn intensifies assortative mating, and so on. This cycle allows substantial isolation to evolve rapidly in the standing variation treatment, leading to significant differences between standing varation and new mutation treatments early in

simulations. The interaction pattern we observed is of interest because speciation in nature is expected to involve multiple isolating barriers (Coyne and Orr 2004). Theories of sympatric speciation typically require both prezygotic and postzygotic isolating barriers for complete reproductive isolation to evolve (e.g., Dieckmann and Doebeli 1999; Fry 2003; Maynard Smith 1966). Empirically, multiple barriers have been observed to separate species in a number of geographic contexts; for example, prezygotic, premating postzygotic, and postmating postzygotic isolating mechanisms have been documented between sympatric *Mimulus* sister species (*M. lewisii* and *M. cardinalis*) (Ramsey et al. 2003), as well as between allopatric *Drosophila* sister species (*D. simulans* and *D. mauritiana*) (e.g., Hollocher and Wu 1996; Price et al. 2000; Watanabe and Kawanishi 1979). Given the potential prevalence of multiple isolating barriers in nature, standing variation could play a significant role in speciation by generating interaction effects similar to those that we observed in our study. Little previous work has focused on the effect of interactions among barriers on the evolution of reproductive isolation (but see, for example, Agrawal et al. 2011; Groot et al. 2010; Nosil and Yukilevich 2008; Widmer et al. 2009); our results suggest that this is an important area of future study.

Finally, mutational covariance affected the evolution of reproductive isolation in the standing variation and new mutation treatments in a number of ways. In simulations with positive mutational covariance that included all isolating mechanisms, standing variation replicates accumulated high levels of reproductive isolation even earlier than in the zero-covariance simulations (Figure 4.3). In the positive covariance scenario the direction of maximum genetic variance (i.e., g_{max} [Schluter 1996]) was parallel to the line separating phenotypic optima. Previous research has demonstrated that when g_{max} is in line with the

direction of selection, adaptation occurs rapidly (Schluter 1996). Thus it is perhaps unsurprising that reproductive isolation also accumulated rapidly with g_{max} oriented in the "right" direction and multiple isolation barriers present. However, the effect of positive covariance is not always this straightforward. In simulations that included positive mutational covariance and BDMIs, but no mate preference, positive covariance suppressed the evolution of reproductive isolation in both the standing variation and new mutation treatments for the duration of the simulations (Figure 4.3). This is likely because positive covariance between traits tends to generate individuals with phenotypes distributed along the line separating phenotypic optima. In the absence of mate preference to generate assortative mating between individuals with similar trait values, gene flow along this distribution restricts population divergence, and BDMIs cannot accumulate.

In the case of negative mutational covariance, we also observed a suppression of isolation in no-preference simulations (Figure 4.4), though likely for a somewhat different reason than described above. Negative covariance between traits tends to generate individuals distributed in a direction perpendicular to the line separating phenotypic optima, meaning that population-level trait change in response to selection is initially skewed away from optimum in the direction of g_{max} (Schluter 1996). In the absence of mate preference to initiate assortative mating, and in the face of ongoing migration, populations in this simulation scenario did not adapt to distinct phenotypic optima, remaining instead in the valley between peaks. Without divergence BDMIs could not accumulate, and reproductive isolation could not evolve.

In negative mutational covariance simulations with preference and without BDMIs, we observed the greatest difference between standing variation and new mutation treatments

in our study. Specifically, standing variation replicates in this scenario achieved high levels of reproductive isolation, while new mutation replicates did not (Figure 4.4). In the standing variation treatment, though most variation was distributed perpendicular to the line separating phenotypic optima, some small amount of variation existed that was in line with the direction of selection. Mate preference was likely able to initiate divergence in the standing variation treatment by facilitating assortative mating between individuals with variation in the "right" direction, which eventually led to the evolution of isolation. In the new mutation treatment there was not any such variation to act upon, and divergence was inhibited.

There are a number of caveats to keep in mind when considering the results of our study. First, we modeled ecologically relevant traits as quantitative characters, assuming additive effects of alleles on the phenotype. However, allelic dominance at traits under selection could alter the effect of standing variation. For instance, selection is less efficient at removing deleterious alleles from the population when they are recessive, because the heterozygous genotype can mask their negative effects (Hendry 2004). Thus the accumulation of deleterious recessive alleles as standing genetic variation could actually inhibit adaptation to distinct phenotypic optima, thereby constraining the evolution of reproductive isolation between populations. An additional caveat is that mate preference was essential in generating differences between standing variation and new mutation treatments in our study, and thus our results do not apply to species without behavioral isolating mechanisms- representing a sizeable proportion of the tree of life. Finally, we assumed symmetry in both the sizes of diverging populations and the migration rates between them, but this is often not the case in the natural world. Asymmetric migration has been observed

among populations undergoing ecological divergence (e.g., Bolnick et al. 2008), and can affect the evolution of both mate preference (e.g., Servedio and Kirkpatrick 1997) and BDMIs (e.g., Bank et al. 2012). Both prezygotic and postzygotic isolating mechanisms were key in generating differences between treatments in our study, and thus our findings do not necessarily apply to populations that violate these assumptions.

Our results indicate that standing variation interacts with reproductive isolating mechanisms, mutational covariance, and migration to generate complex patterns of the evolution of reproductive isolation over time. In a number of simulation scenarios standing variation facilitated the accumulation of high levels of reproductive isolation at an early stage in the speciation process- a time point where rapid divergence is essential for continued persistence of populations. In addition, in one simulation category the amount of reproductive isolation that evolved after 10,000 generations of strong divergent selection was extremely different in replicates with or without standing genetic variation. The combinations of parameters that produced the greatest differences between standing variation and new mutation treatments in our simulations, such as ongoing migration, multiple isolating barriers, and covariance between traits, are not uncommon in nature. Our results therefore suggest that standing variation has the potential to play an important role in determining the outcome of the speciation process in real-world instances of ecological divergence. In the future, researchers seeking to gain a more complete understanding of the genetic basis of ecological speciation should explore the role of standing variation in the context of other factors that shape population dynamics in the natural world.

Tables

Table 4.1: The 24 simulation scenarios in our study represented different combinations of the following factors: migration, with two possible categories depending on whether migration was allowed to occur between populations; reproductive isolating mechanisms, with four possible categories depending on whether mate preference and BDMIs were included; and degree of mutational covariance, with three possible categories depending on whether there was positive, negative, or zero mutational covariance between traits. Each row of the table describes a particular category and gives relevant parameter values for simulations in that category.

Simulation Category	Description	Parameter Value
Migration	Migration occurs between populations in the divergent selection phase	$m = 0.01$
No migration	Migration between populations does not occur	$m = 0$
Preference	Mate preference occurs in the divergent selection phase	$\alpha = 0.1$
No preference	Mate preference does not occur	$\alpha = 0$
BDMIs	Mutation occurs at BDMI loci in the divergent selection phase	$\mu_{BDMI} = 0.0003$
No BDMIs	Mutation does not occur at BDMI loci	$\mu_{BDMI} = 0$
Positive covariance	Mutations at traits under selection are positively correlated	$r_{\mu} = 0.5$
Negative covariance	Mutations at traits under selection are negatively correlated	$r_{\mu} = -0.5$
No covariance	Mutations at traits under selection are not correlated	$r_{\mu} = 0$

Figures

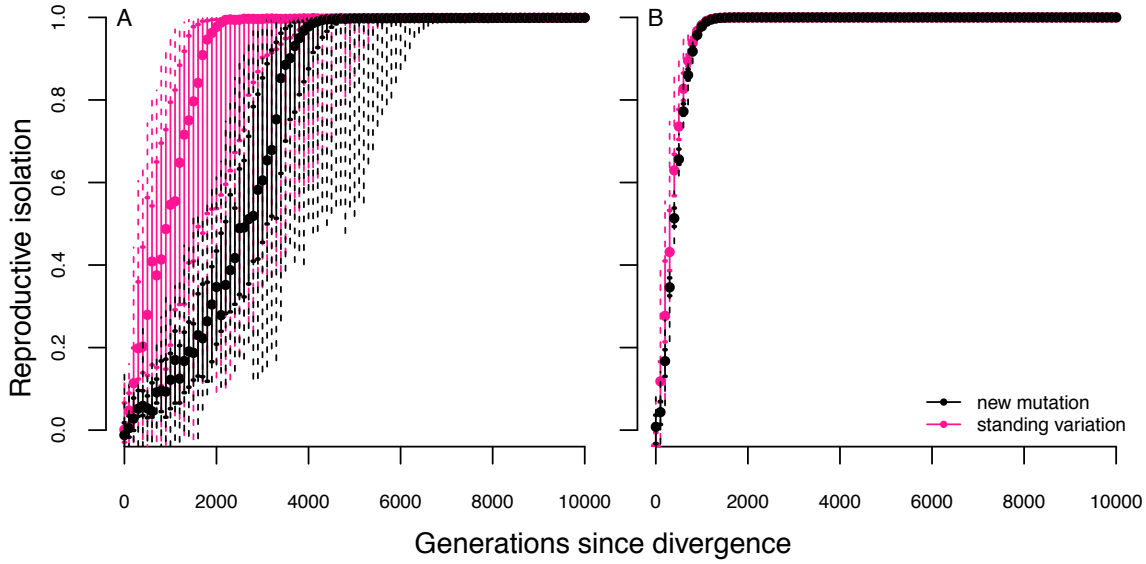

Figure 4.1: Accumulation of reproductive isolation throughout the divergent selection phase in simulations with and without migration. At each data collection time point (i.e., every 100 generations), we measured total reproductive isolation in each of 20 total replicates in the standing variation and new mutation treatments. In this figure and all that follow, dots represent median reproductive isolation among replicates within treatments at a particular time point, with solid lines extending to the first and third quartiles (denoted by horizontal hash marks), and dotted lines extending to minimum and maximum values. Black symbols represent the new mutation treatment, while pink symbols represent the standing variation treatment. Panel A shows values of reproductive isolation in simulations with migration (i.e., $m = 0.01$) and panel B shows isolation in simulations without migration (i.e., $m = 0$); simulations in both panels included all possible isolating mechanisms (i.e., $\alpha = 0.1$ and $\mu_{BDMI} = 0.0003$) in addition to no mutational covariance (i.e., $r_{\mu} = 0$).

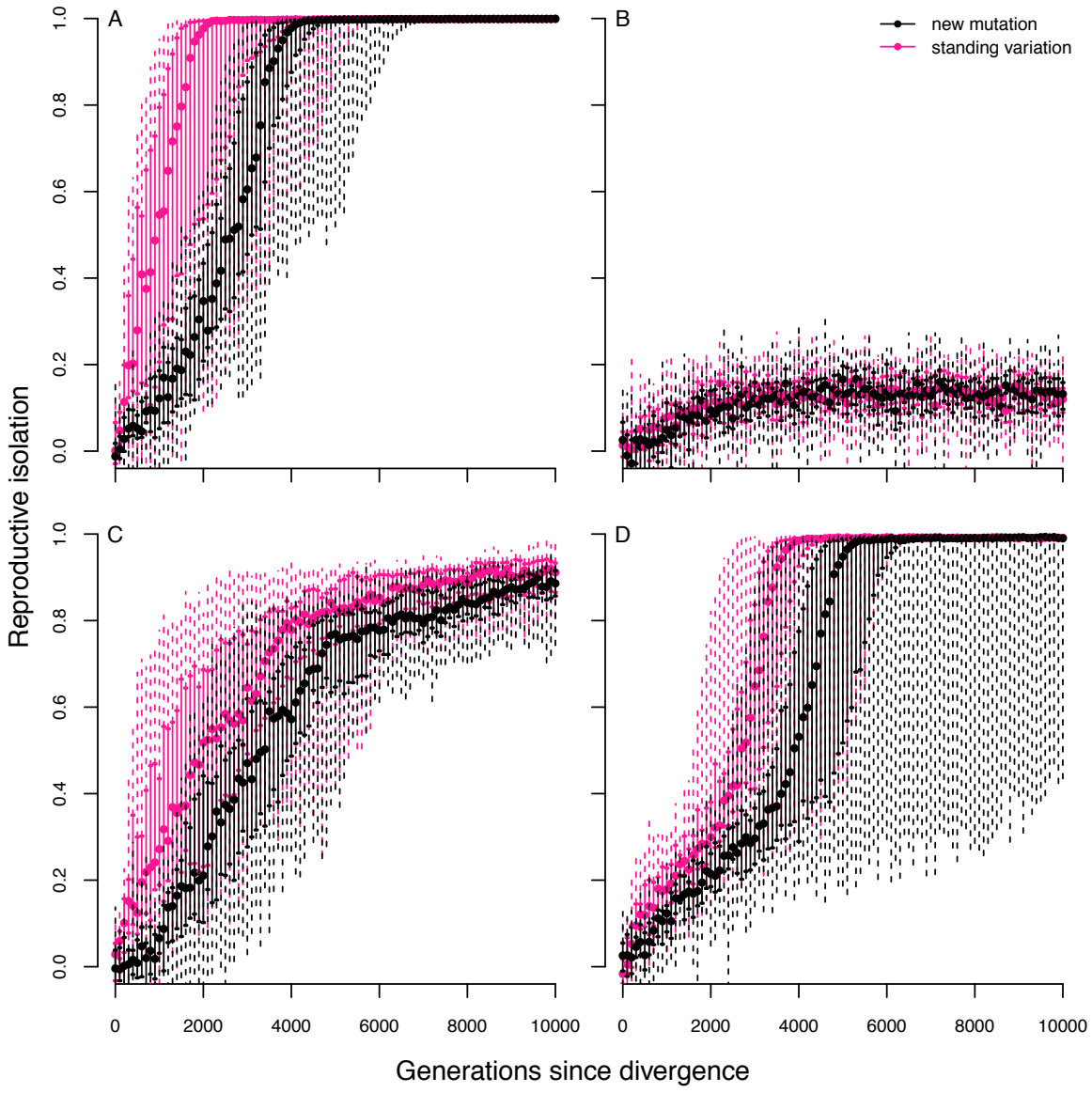

Figure 4.2: Accumulation of reproductive isolation throughout the divergent selection phase in simulations with different reproductive isolating mechanisms. See Figure 4.1 legend for details of plotting; here, panel A shows values of reproductive isolation in simulations with all possible isolating mechanisms (i.e., $\alpha = 0.1$ and $\mu_{BDMI} = 0.0003$), panel B shows isolation in simulations with no mate preference and no BDMIs (i.e., $\alpha = 0$ and $\mu_{BDMI} = 0$), panel C shows isolation in simulations with mate preference and no BDMIs (i.e., $\alpha = 0.1$ and $\mu_{BDMI} = 0$), and panel D shows isolation in simulations with no mate preference and BDMIs (i.e., $\alpha = 0$ and $\mu_{BDMI} = 0.0003$). Simulations in all panels included migration (i.e., $m = 0.01$) in addition to no mutational covariance (i.e., $r_{\mu} = 0.0$).

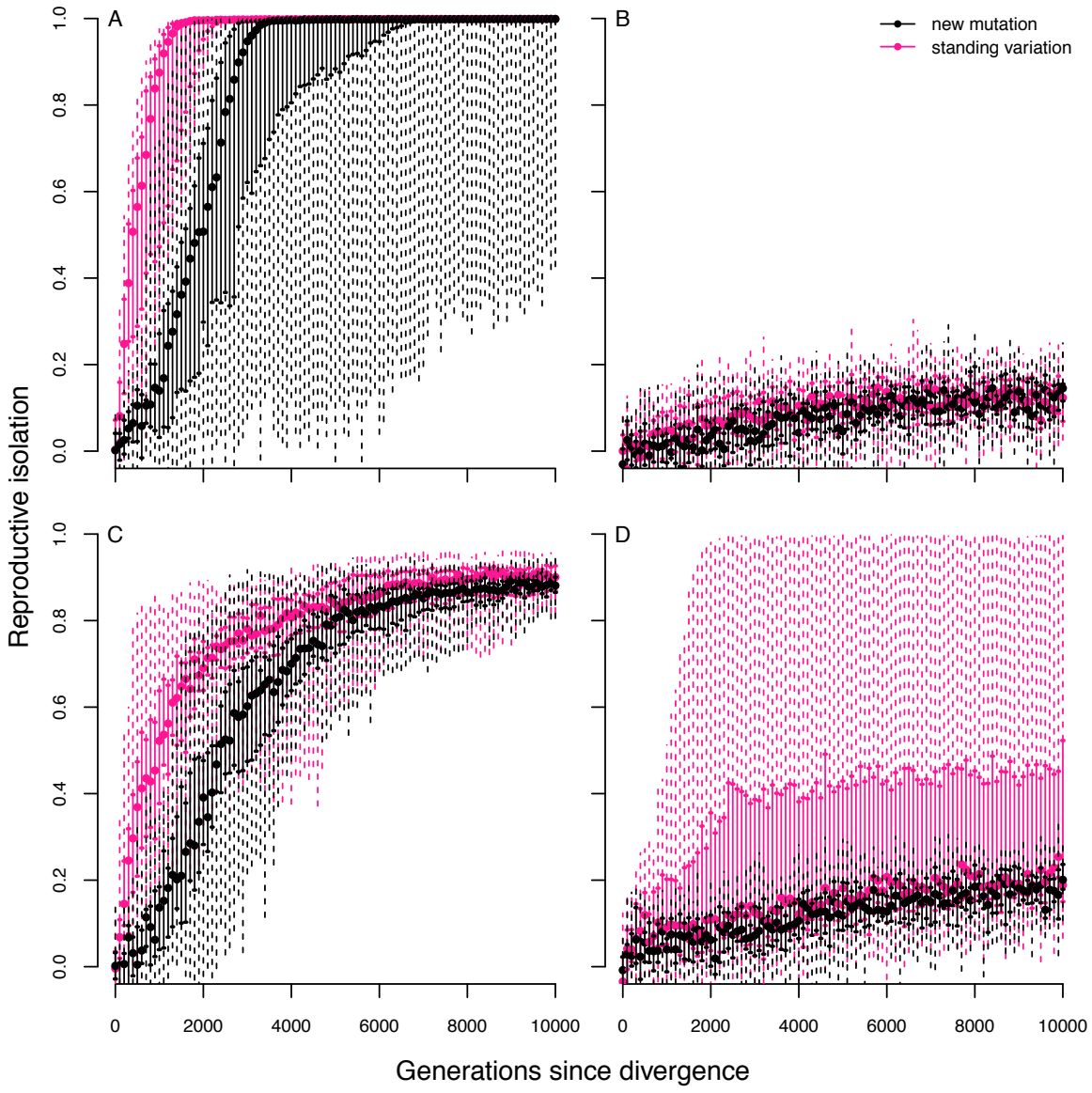

Figure 4.3: Accumulation of reproductive isolation throughout the divergent selection phase in simulations with positive mutational covariance. See Figure 4.1 legend for details of plotting; here, panel A shows values of reproductive isolation in simulations with all possible isolating mechanisms (i.e., $\alpha = 0.1$ and $\mu_{BDMI} = 0.0003$), panel B shows isolation in simulations with no mate preference and no BDMIs (i.e., $\alpha = 0$ and $\mu_{BDMI} = 0$), panel C shows isolation in simulations with mate preference and no BDMIs (i.e., $\alpha = 0.1$ and $\mu_{BDMI} = 0$), and panel D shows isolation in simulations with no mate preference and BDMIs (i.e., $\alpha = 0$ and $\mu_{BDMI} = 0.0003$). Simulations in all panels included migration (i.e., $m = 0.01$) in addition to positive mutational covariance (i.e., $r_\mu = 0.5$).

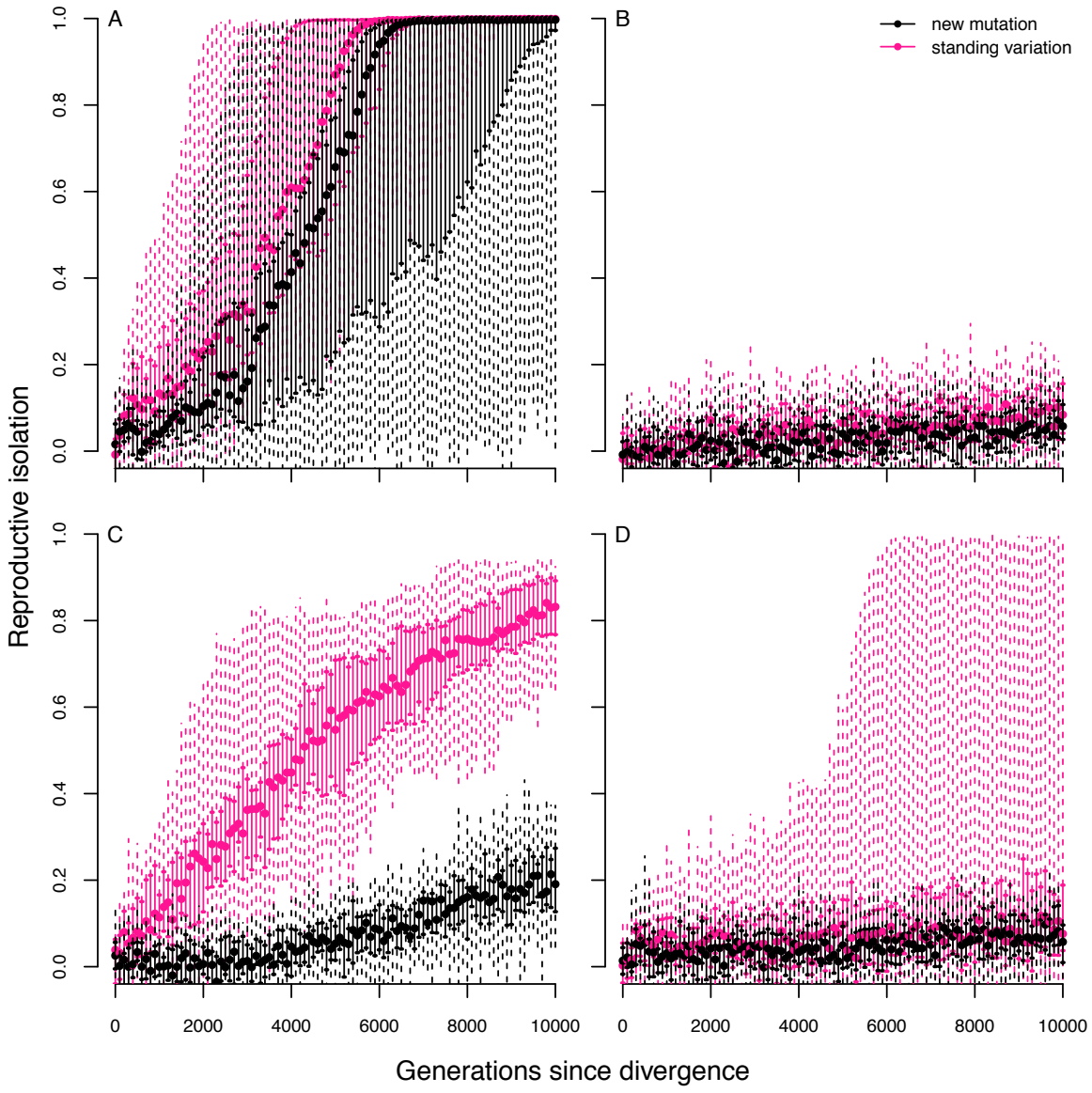

Figure 4.4: Accumulation of reproductive isolation throughout the divergent selection phase in simulations with negative mutational covariance. See Figure 4.1 legend for details of plotting; here, panel A shows values of reproductive isolation in simulations with all possible isolating mechanisms (i.e., $\alpha = 0.1$ and $\mu_{BDMI} = 0.0003$), panel B shows isolation in simulations with no mate preference and no BDMIs (i.e., $\alpha = 0$ and $\mu_{BDMI} = 0$), panel C shows isolation in simulations with mate preference and no BDMIs (i.e., $\alpha = 0.1$ and $\mu_{BDMI} = 0$), and panel D shows isolation in simulations with no mate preference and BDMIs (i.e., $\alpha = 0$ and $\mu_{BDMI} = 0.0003$). Simulations in all panels included migration (i.e., $m = 0.01$) in addition to negative mutational covariance (i.e., $r_\mu = -0.5$).

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CHAPTER V

White Sands Acts as a Barrier to Gene Flow Between Ecologically Distinct Populations in *Aspidoscelis inornata* and *Sceloporus undulatus*

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Abstract

When adaptive divergence occurs in the face of ongoing gene flow, genetic differentiation accumulates between populations as a product of both isolation by ecology and isolation by geographic distance. Factors that affect the relative contributions of ecology and distance to overall levels of genetic differentiation during real-world instances of adaptive divergence remain poorly understood in most systems. Parallel but distinct instances of adaptation by lizards to a novel White Sands habitat provide a window through which we can view the process of adaptive divergence in a comparative framework in order to identify factors that facilitate or inhibit genetic differentiation. Three lizard species have colonized White Sands within the last 10,000 years. Adaptive divergence has occurred with ongoing gene flow in this system, and the degree of divergence between ecologically distinct populations differs among lizard species. To identify factors important for shaping adaptive divergence in the White Sands system, we generated datasets of approximately 30,000 single nucleotide polymorphisms for two White Sands species, *Sceloporus undulatus* and *Aspidoscelis inornata*. We analyzed genetic data with the program BEDASSLE, which determines the relative roles of ecological barriers and geographic distance in generating genetic divergence between populations. We found that local adaptation is an important driver of genetic divergence in both species, and that the magnitude of the effect of White

Sands as a barrier to gene flow is potentially greater for *S. undulatus* than it is for *A. inornata*. A number of factors likely contribute to differences between species in the effect of White Sands on genetic divergence, and we discuss future directions for further investigating dynamics of ecological divergence with gene flow in this system.

Introduction

Despite decades of research, the process by which speciation occurs in the face of gene flow remains relatively poorly understood. Ecological speciation, where divergent natural selection drives the evolution of reproductive isolation between populations (Schluter 1996), is a prominent explanation for speciation in the face of gene flow (Coyne and Orr 2004). However, adaptive divergence with gene flow involves complex interactions between adaptive and neutral processes, and speciation does not always result (Bolnick and Fitzpatrick 2007; Gavrilets 2004). Even when divergent selection acting on populations is strong, the homogenizing effects of ongoing gene flow may inhibit the evolution of complete reproductive isolation (Coyne and Orr 2004; Mayr 1963). Consequently, in some well-studied empirical systems natural selection appears to have driven the evolution of complete reproductive isolation between populations (e.g., *Pundamilia* cichlids [Seehausen et al. 2008] and *Acyrthosiphon* pea aphids [Peccoud et al. 2009]), while in other cases strong selection is associated only with intermediate stages of divergence (e.g., *Geospiza* Darwin's finches [Grant and Grant 2007], *Timema* walking-stick insects [Nosil and Crespi 2004]).

During ecological speciation, genetic divergence accumulates between populations as a product of both isolation by ecology and isolation by geographic distance (Wright 1943). Adaptation to distinct habitats can reduce gene flow via a number of mechanisms, directly

causing genetic differentiation (Schluter and Conte 2009). Geographic distance between populations also effects genetic differentiation, with populations that are farther apart exchanging fewer migrants and exhibiting greater overall divergence (Wright 1943). Though researchers studying adaptive divergence are typically interested in the degree to which local adaptation facilitates speciation, isolation by distance can confound the inference of the effect of particular ecological barriers on genetic differentiation (Smouse et al. 1986). Factors that affect the relative contributions of ecology and geographic distance to genetic divergence during ecological speciation with gene flow are not fully understood.

Populations undergoing rapid adaptive divergence offer a unique opportunity to understand the factors that facilitate or inhibit the accumulation of genetic differentiation in the face of gene flow. This is especially true when a number of lineages undergo adaptation to distinct environments in parallel, because similarities and differences among taxa can be used to determine which key factors are most important for shaping patterns of divergence between populations (Schluter and Nagel 1995). The White Sands system represents an opportunity for understanding the interplay of factors that facilitate divergence in a system where replicated adaptation is occurring over short time scales. White Sands is a vast dune field that formed within the Chihuahuan desert in New Mexico less than 10,000 years ago (Kocurek et al. 2007). The sparsely vegetated white gypsum sand dunes of White Sands represent a striking contrast to the surrounding desert scrubland (referred to here as "dark soils"). Three lizard species (*Sceloporus undulatus*, *Aspidoscelis inornata*, and *Holbrookia maculata*) have colonized White Sands from the dark soils environment (Dixon 1967). Blanched morphs of each species occur in White Sands, while brown morphs occur in dark soils. Blanched color in White Sands is thought to have evolved in response to selection by

avian predators (Rosenblum 2006). Divergence has occurred in parapatry in this system, with ongoing gene flow between populations in White Sands and dark soils environments (Rosenblum 2006; Rosenblum et al. 2007).

The degree of adaptive divergence between ecologically distinct White Sands and dark soils populations differs among species, as evidenced by phenotypic and genetic data. Spectrophotometric data indicate that *H. maculata* exhibit the greatest degree of divergence in body color between White Sands and dark soils populations, while *S. undulatus* exhibit intermediate levels, and *A. inornata* exhibit the lowest degree of divergence. Genetic differentiation between White Sands and dark soils populations is concordant with patterns of divergence in body color; genetic clustering analyses among habitats based on nuclear and mitochondrial DNA demonstrate strong clustering of *H. maculata*, intermediate clustering of *S. undulatus*, and almost no clustering of *A. inornata* (Rosenblum 2006; Rosenblum and Harmon 2011).

Parallel but distinct instances of adaptation to White Sands provide a window through which we can view the process of adaptive divergence in a comparative framework in order to identify factors that facilitate or inhibit the accumulation of genetic differentiation. As a first step in this direction, we developed extensive genetic resources for *S. undulatus* and *A. inornata*, and implemented analyses to understand the relative roles of local adaptation and isolation by distance in generating genetic divergence between populations in each species. We found that local adaptation to divergent environments is an important driver of genetic divergence in both species, and also that local adaptation is potentially less effective at reducing gene flow between White Sands and dark soils populations in *A. inornata* compared with *S. undulatus*. This is consistent with previous findings that *A. inornata* exhibit a lower

degree of overall genetic differentiation than *S. undulatus*. We discuss a number of factors that are likely important in generating observed differences between species in the importance of White Sands as a barrier to gene flow.

Materials and Methods

Sample Collection

We captured *S. undulatus* and *A. inornata* individuals by hand or using a dental floss noose from several different sampling locations within White Sands and dark soils during the summers of 1998, 1999, 2005, 2009, and 2012. We sampled lizards of each species from two different locations within White Sands, located 1.79 kilometers apart. These locations include habitat along the Alkali Flat trail (subsequently referred to as "AF") and habitat along the Backcountry Camping trail (subsequently referred to as "BC"). In addition, we sampled lizards of each species from one location within dark soils habitat: a Bureau of Land Management site northeast of the White Sands Missile Range, Otero County (subsequently referred to as "BLM"). The BLM site is located 86.26 kilometers from AF, and 87.71 kilometers from BC. For *S. undulatus*, we collected 18 total individuals, with nine from White Sands locations (i.e., five from AF and four from BC) and 9 from the BLM dark soils location. For *A. inornata*, we collected 18 total individuals, with nine from White Sands locations (i.e., seven from AF and two from BC) and 9 from the BLM dark soils location.

We collected tissue samples from all individuals. For lizards collected prior to 2012 (i.e., 18 individuals), we stored tissue samples at -80 degrees Celsius in the Museum of Vertebrate Zoology at the University of California, Berkeley. For lizards collected in 2012 (i.e., 18 individuals), we stored tissue in RNAlater (Ambion). Researchers have extracted

high quality genomic DNA from tissue stored in RNAlater in previous studies (e.g., Nsubuga et al. 2004). We followed the tissue preservation protocol suggested by the manufacturer for samples stored in RNAlater. Specifically, we cut tissue into 0.5 centimeter sections and stored tissue, submerged in RNAlater, at 4 degrees Celsius for 10-12 hours to allow RNAlater to penetrate tissue. We subsequently transferred samples to a -20 degrees Celsius freezer for long-term storage. While in captivity, we housed lizards individually in small cages with 12-hour light cycles, and fed them *ad libitum*. After processing, we released lizards at the original points of capture.

Characterization of Genetic Variation

We extracted genomic DNA from tissue samples using a DNeasy Blood and Tissue Kit (Qiagen). In order to obtain sequences for long regions of the genomes of *S. undulatus* and *A. inornata*, we constructed fosmid libraries (BACPAC Resources Center, CHORI) from single individuals of each species with 40kb inserts. We then chose approximately 100 clones for each species corresponding to random locations throughout the genomes of *A. inornata* and *S. undulatus*. We performed a combination of Illumina and 454 sequencing of the selected clones. To clean sequencing reads we first discarded duplicate reads and used a mapping approach to filter PhiX-contaminated reads. We then trimmed low quality ends and combined overlapping reads using FLASH (Magoc and Salzberg 2011). Finally, we created a *de novo* assembly using the program gsAssembler 2.6 (454 Life Sciences 2011), which resulted in approximately 1000 contigs per species located randomly throughout the genome.

We then used sequence capture technology to generate a dataset of sequence variation at the population level. First we submitted a combined library of *S. undulatus* and *A. inornata* contigs to Roche Nimblegen for design of 80-100bp capture probes. The resulting probes covered 96.4% of the original target sequences. Next we generated Illumina TruSeq barcoded libraries for all individuals from each species, performed capture protocols according to the Roche Nimblegen specifications, and sequenced using the Illumina MiSeq PE150 platform. Finally, for each species we mapped reads to reference sequences using default parameters in Bowtie2 (Langmead and Salzberg 2012), and called variants on resulting BAM files using GATK HaplotypeCaller (McKenna et al. 2010). We then filtered variant calls to retain only single nucleotide polymorphisms (SNPs). We also removed variant calls that had extremely high or extremely low levels of coverage, and filtered reads based on mapping quality.

Statistical Analysis

We calculated summary statistics for our genetic datasets for each species. Specifically, we used the program VCFtools (Danecek et al. 2011) to determine mean nucleotide diversity across loci within White Sands and dark soils populations, and mean F_{ST} across loci between White Sands and dark soils populations. We pooled genetic data for individuals from White Sands sampling localities (i.e., AF and BC sites) to calculate summary statistics because we are interested in comparing patterns of divergence between ecologically distinct White Sands and dark soils habitats. To further describe genetic differentiation between populations, we conducted principal components analyses (PCAs) on the genetic data, using codes to represent diploid genotypes as quantitative characters. A

code of 0 represented the homozygote of the most common allele, 1 represented heterozygotes, and 2 represented the homozygote of the least common allele. We coded genotypes for all individuals at all loci, and performed PCAs on the resulting datasets for each species using R vers. 3.1.2 (R Development Core Team 2014).

We used the program BEDASSLE (Bradburd et al. 2013) to estimate the proportion of divergence between White Sands and dark soils populations attributable to local adaptation. BEDASSLE is a program that simultaneously assesses the degree of genetic differentiation between populations caused by ecological distance (i.e., habitat) and geographic distance. Specifically, BEDASSLE models allele frequencies at a set of loci as spatially correlated Gaussian processes, and measures the strength of covariance in allele frequencies between populations of varying geographical and ecological distances from one another. BEDASSLE uses a Bayesian framework implemented as a Markov chain Monte Carlo algorithm to estimate a number of model parameters, including the effect sizes of ecological and geographic distance on the covariance between allele frequencies across populations. BEDASSLE returns values for the estimated ratio of genetic differentiation attributable to ecological distance compared with geographic distance, denoted as α_E/α_D.

We ran BEDASSLE using data for all variable sites, with AF, BC, and BLM sampling localities included in the analysis as separate geographic populations. In addition, we included White Sands as a hypothesized barrier to gene flow, coded as a binary variable in the environmental distance matrix. We ran BEDASSLE separately for each species for 500,000 generations, sampling a total of 80,000 times. We used the default exponential priors on α_E and α_D, and adjusted tuning parameters according to the specifications of the BEDASSLE manual to ensure that acceptance rates stayed within the recommended window

of between 20% and 70%. We assessed convergence visually using trace plots, and calculated effective sample sizes from the posterior distributions of the likelihood functions using the R package coda (Plummer et al. 2006). In addition, we applied a posterior predictive approach to assess model adequacy. To do this, we used the *posterior.predictive.sample* function in BEDASSLE to generate expected values of F_{ST} between populations based on parameter values from our MCMC runs. We simulated 100 posterior predictive datasets and compared them to our observed values of F_{ST} to ensure that the model parameter estimates generated by BEDASSLE could sufficiently predict relationships between populations in terms of genetic divergence based on geographic and ecological distance.

We compared posterior distributions of the ratios of α_E/α_D in *A. inornata* and *S. undulatus* in order to understand the magnitude of the effect of local adaptation on genetic divergence in each species. Specifically, we discarded all but the last 25% of sampled generations as burn-in. We then randomly drew values of α_E/α_D from the posterior distributions for *A. inornata* and *S. undulatus* and calculated the difference between the values for the two species. We repeated this process 10,000 times, and determined the proportion of comparisons where difference values were greater than (or less than) zero in order to determine whether species typically differed in inferred ratios of α_E/α_D. We also calculated median values and 95% credible intervals for α_E/α_D for each species. We performed BEDASSLE and coda analyses in R vers. 3.1.2 (R Development Core Team 2014).

Results

After data cleaning and processing, our analysis produced 289 regions of contiguous sequence (i.e., contigs) located randomly throughout the genome in *S. undulatus*, 237 of which contained sites that were variable among sequenced individuals. Contigs with variation ranged from 2,064-38,462bp in length. We detected 32,080 SNPs across all contigs, and the median number of variable sites per contig was 71 (minimum = 1; maximum = 790). Our analysis of genetic data for *A. inornata* produced 363 contigs, 304 of which contained sites that were variable among individuals. Contigs with variation ranged from 2,024-46,253bp in length. We detected 20,968 SNPs across all contigs. Median number of variable sites per contig for *A. inornata* was 43 (minimum = 1; maximum = 510).

Mean nucleotide diversity across loci was 0.18 for *S. undulatus* and 0.24 for *A. inornata*. F_{ST} values between White Sands and dark soils populations were similar for the two species (i.e., 0.10 and 0.09 for *S. undulatus* and *A. inornata*, respectivley). Results of the PCA for *S. undulatus* indicated that PC1 explained 23.3% of variation in the genetic dataset, and PC2 explained 8.3%. The proportion of variation explained by subsequent PCs ranged from 6.1% to 3.9% each, with 15 PCs required to account for greater than 95% of variation within the dataset. PCA results were similar for *A. inornata*, with PC1 explaining 19.0% of variation and PC2 explaining 8.5%. Subsequent PCs explained between 7.0% and 3.2% each, with 16 PCs required to account for greater than 95% of total variation. For each species, between-habitat variation corresponded to PC1, with AF and BC individuals clustering together and BLM individuals clustering together (Figures 5.1 and 5.2). Within-habitat variation corresponded to subsequent PCs. In general, *S. undulatus* exhibited less within-

habitat variation along PC2 for samples from the White Sands locations compared with the dark soils location (Figure 5.1), while the opposite was true for *A. inornata* (Figure 5.2).

Effective samples sizes calculated from the posterior distribution of the likelihood function generated by BEDASSLE were 72 for *A. inornata* and 37 for *S. undulatus*. Posterior predictive values of F_{ST} fit the observed values relatively well, although predicted F_{ST} values were generally somewhat higher than observed values for each species. We observed median α_E/α_D values of 17.04 (95% credible interval [0.75, 107.69]) for *A. inornata*, and 66.29 (95% credible interval [2.44, 371.16]) for *S. undulatus*, meaning that ecological distance contributed to genetic divergence in both species. In 78% of pairwise comparisons of values drawn from the posterior distributions of each species, α_E/α_D was higher for *S. undulatus* than *A. inornata*.

Discussion

We detected evidence of genetic divergence between ecologically distinct White Sands and dark soils populations of *A. inornata* and *S. undulatus*. In addition, our results provide conclusive evidence that White Sands is an important factor in generating genetic divergence independent of geographic distance, and indicate that the magnitude of the effect of White Sands as a barrier to gene flow could be greater for *S. undulatus* than it is for *A. inornata*. We discuss each of these results below, along with future directions for further investigating factors underlying observed differences between species.

Our summary statistics revealed genetic divergence between ecologically distinct populations in both species. We observed F_{ST} values of 0.10 and 0.09 for *S. undulatus* and *A. inornata*, respectively, indicating some demographic structure between White Sands and dark

soils populations. Similarity in F_{ST} values between species is consistent with results of previous analyses of different nuclear datasets for this system (i.e., 19 anonymous loci with 207 SNPs for *S. undulatus* and 47 AFLP markers for *A. inornata*) (Rosenblum and Harmon 2011). The results of our PCAs indicate that divergence between White Sands and dark soils populations accounts for the greatest proportion of variation in the genetic datasets, with subsequent PCs corresponding to within-habitat variation (Figures 5.1 and 5.2). Thus in both *S. undualuts* and *A. inornata*, individuals from different habitats exhibit more genetic differentiation than those from within the same habitat.

For both species, we found evidence that ecological distance between populations (i.e., whether populations inhabited White Sands or dark soils habitat) was an important factor in generating genetic divergence. In BEDASSLE analyses, we detected median α_E/α_D values of 66.29 for *S. undulatus* and 17.04 for *A. inornata*. α_E/α_D compares the magnitude of the effect of the specified ecological variable to that of geographic distance in contributing to genetic divergence between populations. Thus for *S. undulatus*, White Sands was responsible for generating a degree of genetic divergence equivalent to that of 66.29 kilometers of geographic distance, and for *A. inornata*, White Sands was responsible for generating divergence equivalent to 17.04 kilometers of geographic distance. In other words, the genetic divergence between White Sands and dark soils populations is equivalent to what would be expected for populations in the same habitat if the geographic distance between them were increased by 66.29 kilometers for *S. undulatus*, and 17.04 kilometers for *A. inornata*.

A limited number of previous studies have employed BEDASSLE to examine the effects of ecological barriers on genetic divergence in natural populations, with variable results. Barley (2014) found that in *Eutropis multifasciata*, ocean channels that have

separated populations on different islands for less than 20,000 years have the effect on genetic divergence of approximately 460 kilometers of geographic distance. Harvey and Brumfield (2014) found that mountain ranges separating *Xenops minutus* populations had an effect on genetic divergence comparable to 2,500 kilometers of geographic distance. Finally, Gray et al. (2014) found that in *Andropogon gerardii*, climate-related differences that have existed between habitats for 10,000 years generate genetic divergence equivalent to approximately 50 kilometers of geographic distance. These groups are taxonomically quite different from one another, and it is therefore difficult to compare out results with those of previous studies. However, it is interesting to note that our results generally fall in line with α_E/α_D ratios in populations separated by recent barriers- especially considering White Sands formed more recently than any of the barriers considered in previous studies.

In addition, values of α_E/α_D tended to differ between species, suggesting that the magnitude of the effect of ecology on genetic divergence might be larger for *S. undulatus* than *A. inornata*. This was expected given results of previous research in the White Sands system have indicated that *A. inornata* exhibit a lower overall degree of genetic clustering within habitats relative to *S. undulatus* (Rosenblum 2006; Rosenblum and Harmon 2011). A number of factors related to ongoing gene flow between populations could be responsible for the observed differences in α_E/α_D ratios between species. Perhaps most relevant, *A. inornata* and *S. undulatus* exhibit substantial differences in dispersal capabilities and population connectivity. *A. inornata* are active foragers with high dispersal rates (Persons 2005) and populations that are continuously distributed across the ecological transition zone from White Sands into dark soils (Rosenblum 2006). In contrast, *S. undulatus* are territorial ambush predators with small home range sizes (Haenel et al. 2003), whose populations are more

patchily distributed across the ecological transition from White Sands to dark soils (Rosenblum 2006). It seems possible that the high dispersal capability of *A. inornata* has led to a greater degree of migration between ecologically distinct habitats, reducing the effectiveness of White Sands as a barrier to gene flow in this species.

Another potential factor that could contribute to differences in α_E/α_D values between species is mate preference. Assortative mating within ecologically distinct populations is predicted to accelerate the evolution of reproductive isolation (Felsenstein 1981), and could therefore influence the magnitude of the effect of ecological barriers in generating genetic divergence. Previous research has demonstrated asymmetrical mate preference in *S. undulatus*, where White Sands males exhibit a preference for local mates, while dark soils males do not (Hardwick et al. 2013). Preference for local mates, even if it is asymmetrical in nature, could reduce gene flow between White Sands and dark soils *S. undulatus*, thereby increasing the importance of White Sands as a factor facilitating genetic divergence in this species. Future research on mate preference in *A. inornata* will be important for understanding whether preference plays a role in generating distinct patterns of divergence among lizard species in the White Sands system.

We detected differences between observed values of F_{ST} in our dataset and posterior predictive F_{ST} values generated by BEDASSLE based on inferred model parameters. BEDASSLE assumes that all populations exhibit the same amount of variance in allele frequencies, and violations of this assumption, including local differences in population size, inbreeding, historical bottlenecks, and population substructure can result in discordance between posterior predictive and observed F_{ST} values (Bradburd et al. 2013). In particular, our dataset likely violates the population size assumption because for both species the

population at White Sands is small relative to the surrounding dark soils populations (Rosenblum 2007). In addition, previous research has detected evidence of a population bottleneck at White Sands in *S. undulatus* (Rosenblum 2007), and *A. inornata* likely experienced a similar bottleneck upon colonization of White Sands. Posterior predictive and observed values of F_{ST} differed similarly in both magnitude and direction in *A. inornata* and *S. undulatus*, indicating that while values of α_E/α_D are potentially skewed due to violations of the assumptions of the model, between-species comparisons of aE/aD are unlikely to be substantially affected.

One shortcoming of our study is the limited number of dark soils sampling sites, and the fact that relative distance between White Sands and dark soils sampling sites is unequal. To improve on our sampling design we collected and are currently generating data for additional *S. undulatus* and *A. inornata* from a dark soils location approximately 16 kilometers from the White Sands sampling sites, as well as a number of samples from lizards distributed along the ecological transition zone between White Sands and dark soils habitat. An additional drawback of our study design is that BEDASSLE assumes all loci included in the analysis are unlinked, whereas linkage exists in our dataset among variable sites within contigs. Linkage among sites has the effect of reducing our overall sample size, which likely reduces our ability to infer fine scale patterns of isolation by ecology and isolation by distance. To improve upon this aspect of our study we are currently using restriction-site associated DNA sequencing (RADSeq) to generate genetic data for samples collected in 2012 and 2013, as well as for samples from our original White Sands and dark soils sites. The RADSeq dataset will help us overcome the obstacle of non-independence among sites by providing a large number of putatively unlinked loci for subsequent BEDASSLE analysis.

This study represents a first step in understanding how different factors affect adaptive divergence with gene flow in the White Sands system. We have demonstrated that White Sands (though recently formed) is a significant factor in generating genetic divergence between populations in the face of ongoing gene flow, independent of geographic distance. In addition, we have demonstrated that White Sands likely varies in the effectiveness with which it facilitates genetic divergence between populations with respect to different species. A number of the factors that play potentially important roles in generating observed differences between *A. inornata* and *S. undulatus*, including population structure, mate preference and the genetic architecture of traits under selection, can be used to make clear, species-specific predictions about the dynamics of ongoing gene flow in this system. Future research will examine two-way patterns of migration between White Sands and dark soils populations in *A. inornata* and *S. undulatus* to further disentangle the roles of different factors in generating patterns of divergence and to determine which key factors likely contribute to observed differences in the effectiveness of White Sands as an ecological barrier to gene flow.

Figures

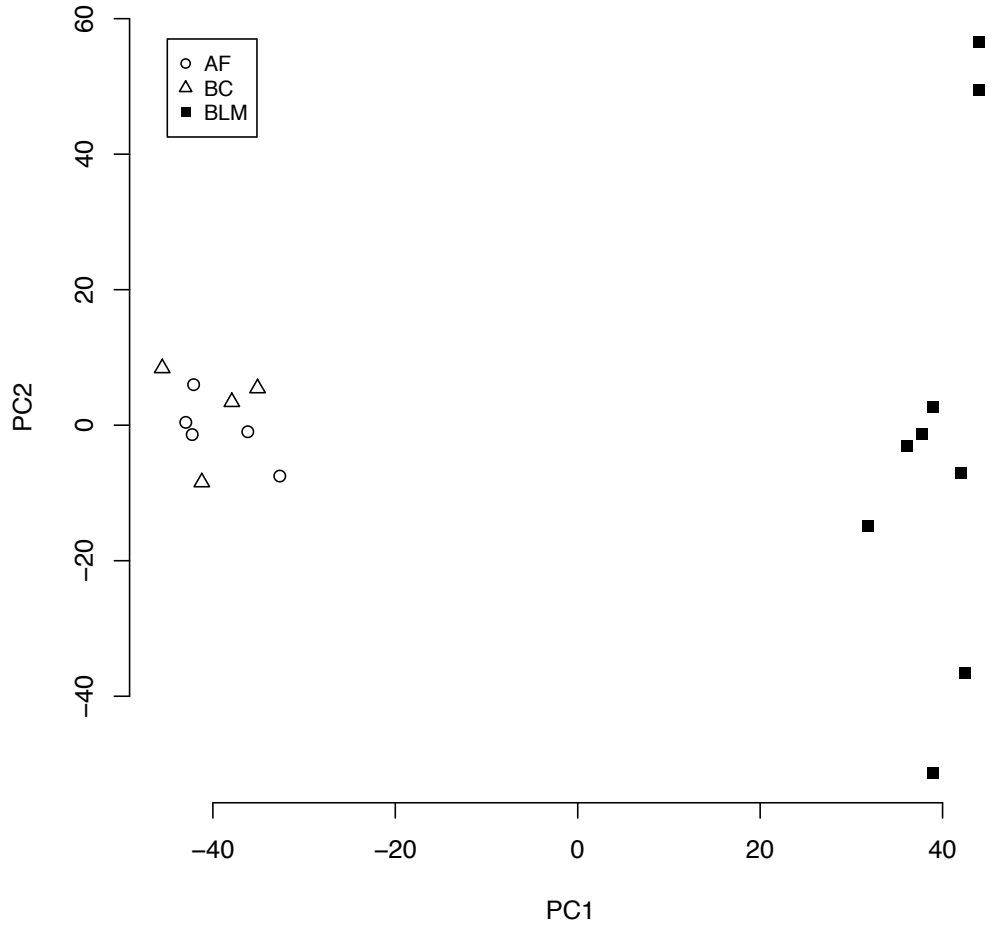

Figure 5.1: Results of principal components analysis on genetic data coded as quantitative traits for *S. undulatus*. Unfilled symbols represent individuals from White Sands sampling sites, with circles representing AF lizards and triangles representing BC lizards. Filled squares represent individuals from the dark soils BLM site. PC1, which corresponds to genetic differentiation between populations in ecologically distinct White Sands and dark soils habitats, is plotted along the x-axis. PC2, which corresponds to within-habitat genetic variation, is plotted along the y-axis. In *S. undulatus*, variation in PC2 was greater among individuals from dark soils compared with those from White Sands.

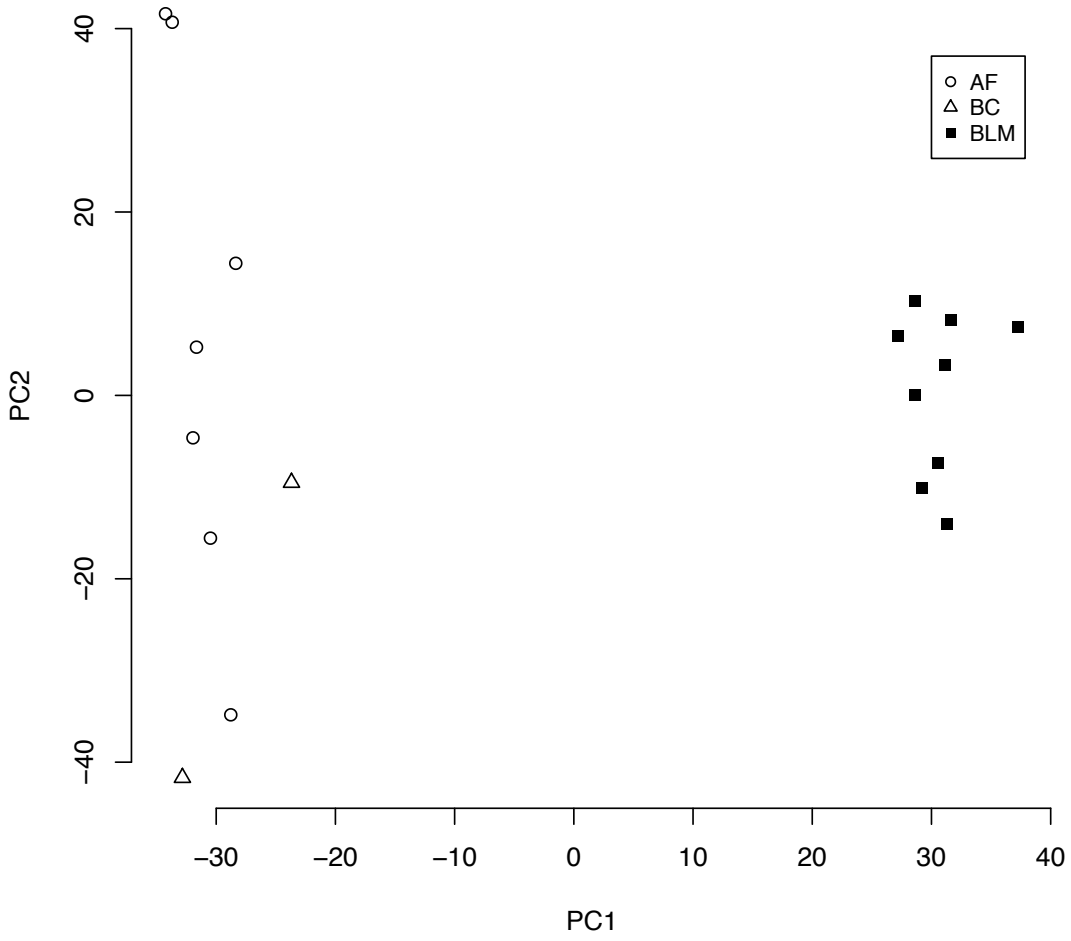

Figure 5.2: Results of principal components analysis on genetic data coded as quantitative traits for *A. inornata*. Unfilled symbols represent individuals from White Sands sampling sites, with circles representing AF lizards and triangles representing BC lizards. Filled squares represent individuals from the dark soils BLM site. PC1, which corresponds to genetic differentiation between populations in ecologically distinct White Sands and dark soils habitats, is plotted along the x-axis. PC2, which corresponds to within-habitat genetic variation, is plotted along the y-axis. In *A. inornata*, variation in PC2 was greater among individuals from White Sands compared with those from dark soils.

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