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SPECIAL ISSUE: THE MOLECULAR MECHANISMS OF ADAPTATION AND SPECIATION: INTEGRATING GENOMIC AND MOLECULAR APPROACHES

Survival by genotype: patterns at *Mc1r* are not black and white at the White Sands ecotone

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Abstract

Measuring links among genotype, phenotype and survival in the wild has long been a focus of studies of adaptation. We conducted a 4-year capture–recapture study to measure survival by genotype and phenotype in the Southwestern Fence Lizard (*Sceloporus cowlesi*) at the White Sands ecotone (transition area between white sands and dark soil habitats). We report several unanticipated findings. First, in contrast with previous work showing that cryptic blanched coloration in *S. cowlesi* from the heart of the dunes is associated with mutations in the melanocortin-1 receptor gene (*Mc1r*), ecotonal *S. cowlesi* showed minimal association between colour phenotype and *Mc1r* genotype. Second, the frequency of the derived *Mc1r* allele in ecotonal *S. cowlesi* appeared to decrease over time. Third, our capture–recapture data revealed a lower survival rate for *S. cowlesi* individuals with the derived *Mc1r* allele. Thus, our results suggest that selection at the ecotone may have favoured the wild-type allele in recent years. Even in a system where a genotype–phenotype association appeared to be black and white, our study suggests that additional factors – including phenotypic plasticity, epistasis, pleiotropy and gene flow – may play important roles at the White Sands ecotone. Our study highlights the importance of linking molecular, genomic and organismal approaches for understanding adaptation in the wild. Furthermore, our findings indicate that dynamics of natural selection can be particularly complex in transitional habitats like ecotones and emphasize the need for future research that examines the patterns of ongoing selection in other ecological 'grey' zones.

Keywords: adaptation, capture–recapture, crypsis, ecotone, JAGS, natural selection

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Introduction

Measuring natural selection in the wild has been a central focus of research in evolutionary biology since the turn of the last century (e.g. Weldon 1901; Di Cesnola 1907). Evolutionary biologists in the Modern Synthesis such as Dobzhansky (1947) and Haldane (1954) were among the first to consider how the link between genotype and phenotype would affect dynamics of selection (e.g. reviewed in Endler 1986). More contemporary efforts have focused on measuring changes in heritable traits over time (e.g. Grant & Grant 1989; Barrett *et al.* 2008; Linnen & Hoekstra 2009) and correlating individual survivorship to phenotype using capture–recapture methods (e.g. Brodie 1992; Zabel *et al.* 2005; Gimenez *et al.* 2006).

Whereas early capture–recapture studies were primarily concerned with population size and treated survival probability as a nuisance parameter (e.g. Lebreton *et al.* 1992), survival is now the focus of capture–recapture models that estimate natural selection in the wild (e.g. Brodie 1992; Gimenez *et al.* 2006, 2009; Cam 2009). Selection studies employing capture–recapture

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aim to correlate survivorship with specific trait values by uniquely marking individuals and recapturing them through time (Lebreton *et al.* 1992; Kingsolver & Smith 1995; Borchers *et al.* 2002; Cam 2009). The capture–recapture framework accounts for our imperfect ability to observe individual animals, which (if unaccounted for) can confound the signature of selection in the wild (Gimenez *et al.* 2009). Moreover, recent analytical advances including the use of a Bayesian framework to fit population models (Gimenez *et al.* 2006, 2009; Kéry & Schaub 2012; Lloyd *et al.* 2014; Zipkin *et al.* 2014) now allow capture–recapture data to be more robustly applied to understanding dynamics of natural selection in the wild.

White Sands in southern New Mexico provides an ideal setting to study the effects of natural selection on both phenotype and genotype. White Sands is a geologically young and ecologically novel habitat composed of 650 km^2 of gypsum sands that have been deposited over the last 2000–7000 years (Langford 2003; Kocurek *et al.* 2007). A number of species exhibit local adaptation to the gypsum sand dunes. Most notably, three species of lizard exhibit convergence for a number of traits at White Sands. White Sands populations exhibit morphological and behavioural differences compared to their closely related conspecifics in the surrounding Chihuahuan Desert (Des Roches *et al.* 2011, 2015a, Des Roches *et al.* 2015b; Rosenblum & Harmon 2011; Robertson *et al.* 2011; Hardwick *et al.* 2013). The most notable difference between White Sands and dark soils lizards is that White Sands populations exhibit blanched coloration (Rosenblum 2006; Rosenblum *et al.* 2010; Rosenblum & Harmon 2011). Blanched coloration in all three lizard species is associated with mutations in the melanocortin-1 receptor gene (*Mc1r*), a key gene in the vertebrate melanin synthesis pathway (Rosenblum *et al.* 2004). For two of the three White Sands lizard species, functional assays have confirmed that the observed mutations at *Mc1r* have functional effects (Rosenblum *et al.* 2010), and population genomic analyses have revealed strong evidence for natural selection at *Mc1r* and the surrounding chromosomal region (Laurent *et al.* 2016). Thus, studies of selection at the population level at White Sands can address dynamics of change at both phenotypic and genotypic levels.

We performed a capture–recapture study to measure patterns of natural selection on genotype and phenotype of an open population of Southwestern Fence lizards (*Sceloporus cowlesi*) at the White Sands ecotone. We chose to conduct this study at the ecotone – the transition area between White Sands and dark soils habitat – for several reasons. Ecotones are transitional zones that can share biotic and abiotic characteristics of two neighbouring habitats (e.g. Wiens 1992). Further, they may represent distinctive or intermediate selective environments on a microgeographic scale (e.g. Richardson *et al.* 2014). For example, the White Sands ecotone has mostly gypsum substrate (so ecotonal lizards are expected to be relatively light in coloration), but is more densely vegetated and has a higher diversity of potential predators and interspecific competitors compared to the heart of the dunes (S. Des Roches, personal observation). The transition between white sand and dark soil substrate is fairly abrupt, but fence lizards are found in both habitats in reasonably close proximity. As a result, the interplay between selection and gene flow is also particularly dynamic in ecotonal populations, and previous research has documented gene flow between White Sands and dark soil populations of *S. cowlesi* (Rosenblum 2006; Rosenblum *et al.* 2007; Laurent *et al.* 2016). Finally, most of our prior work on *Mc1r* and colour variation has focused on *S. cowlesi* populations in the heart of the dunes (Rosenblum *et al.* 2004, 2010; Laurent *et al.* 2016), and comparatively little is known about genotypic and phenotypic variation at the ecotone. *Mc1r* allele frequency data for *S. cowlesi* at the White Sands ecotone are only published from a small number of individuals ($N < 20$ individuals) collected nearly a decade ago (Rosenblum *et al.* 2010).

Against this background, the objectives of our study were to (i) understand the relationship between *Mc1r* genotype and colour phenotype for *S. cowlesi* at the White Sands ecotone, (ii) compare past data on *Mc1r* allele frequency at the ecotone to a much larger contemporary sample and (iii) use capture–recapture models in a Bayesian framework to estimate survivorship by both colour phenotype and *Mc1r* genotype over a 4-year study period. Linking genotype, phenotype and survival in natural populations remains difficult, so a secondary objective of our study was to provide a novel application of capture–recapture models to understanding both survival by phenotype and genotype in the wild.

Methods

Sampling methods

We performed a capture–recapture study on *Sceloporus cowlesi* inhabiting the southeastern ecotone of White Sands, New Mexico. The ecotone has white gypsum substrate but is typically more vegetated and contains a higher density of avian and terrestrial predators and competitors than the heart of the White Sands dune field (S. Des Roches, unpublished data). We exhaustively captured all lizards we observed in a single area of nearly 10.7 ha, which extended approximately 200 m from the ecotone westward into White Sands (UTM:

13S 388460-388640 mE, 3625250-3725780 mN). Lizards were primarily associated with vegetation in large 'interdune' areas, which were enclosed by 3 to 10-m-high dunes. We captured male and female individuals by noose or by hand between 07:00 and 15:00 and returned all lizards during daylight hours after measuring, identifying and uniquely marking them. We sampled twice in 2012, both early (May–June) and late (July–August) in the activity season. For subsequent years (2013–2015), we sampled once a year early (May–June) in the activity season. We collected genetic samples via nondestructive buccal swabbing in the last 3 years. Our sampling team consisted of two to four people, and we recorded person-hours spent in each interdune to quantify and maintain consistent sampling effort.

Measuring phenotype

We measured brightness of the dorsal midline using digital photographs. We photographed lizards under standard conditions (see Stevens *et al.* 2007) using a Nikon D5100 with either a 28- or 50-mm lens at a shutter speed of 1/160 and 100 ISO after lizards were warmed in a basking tank for 2 min. We placed lizards on white poster board with a white, grey and dark grey standard (Adorama QPcard 101). We used methods described in Stevens *et al.* (2007) to extract brightness from photographs. First, we standardized colours by linearizing and equalizing each photograph using the colour standards in each photograph and the curves function in Adobe Photoshop CS6. Next, we took red, green and blue values (8 bits/Channel Adobe RGB colour space with maximal brightness of 765) using the eyedropper tool in Photoshop after averaging the colour over a 2–4 mm^2 centred on the dorsal midline. Finally, we summed the RGB values to get an estimate of brightness. We also collected spectrophotometric data (following Rosenblum 2006), but we experienced an unanticipated solarization of our reflectance probe, which increased noise in lower wavelengths. We used a subset of the spectrophotometric data (excluding noise) to confirm that brightness calculated from photographs and from spec data were significantly correlated (linear regression: $R^2 = 0.47$, $t = 15.3$, $P \ll 0.001$). For simplicity, we present results only from photographic analysis here.

Measuring genotype

To determine *Mc1r* genotype, we took buccal swabs for DNA analysis the first time each individual was captured. We used swabs from an Isohelix kit with dricapsules and later extracted genomic DNA from swabs using the Isohelix Xtreme DNA Kit (Cell Projects, Kent, UK) using the manufacturer's protocol. We then used 25 μL PCRs that included 12.5 μL KAPA HiFi HotStart ReadyMix, 0.75 μL forward *Mc1r* primer (10 μM), 0.75 μL reverse *Mc1r* primer (10 μM), 9 μL nuclease-free PCR buffer and 2 μL DNA template (*Mc1r* primers following Rosenblum *et al.* 2004). PCR conditions included 2-min denaturation step at 95 °C, followed by 35 amplification cycles of 30 s 95 °C, 30 s annealing at 61 °C and extension of 100 s at 68 °C, with final extension at 68 °C for 2 min. After PCR amplification, we purified PCR products using ExoSAP-IT (Affymetrix Inc.) based on the manufacture's protocol. Samples were sequenced at the UC Berkeley DNA Sequencing Facility, and reads were edited and aligned in Sequencher (Gene Codes Co., Ann Arbor, MI, USA). We also aligned sequences against both blanched and wild-type reference sequences (GenBank Accession Nos AY586147 and AY586117, respectively) to obtain genotypes for the *Mc1r* nucleotide position 622, the site that of the causal mutation.

For capture–recapture analyses described below, we took buccal DNA samples and used brightness measurements from only the first instance an individual was captured (individuals were captured between one and four times). As a result, our sample size consisted of 157 genotyped lizards. We used a single-factor ANOVA to determine whether there was a correlation between *Mc1r* genotype and phenotype (brightness of the dorsal midline). We used a Fisher's exact test to determine whether the frequency of derived *Mc1r* alleles changed over the 10 years from 2003–2005 (from Rosenblum *et al.* 2010) to present (2013 and 2014). We ran the test on the total number of derived vs. wild-type alleles on 14 individuals (2N = 28) captured in 2003–2005, 62 (2N = 124) captured in 2013 and 114 (2N = 228) captured in 2014 (~20% of the 2014 samples were recaptures). Additionally, we tested for departures from Hardy–Weinberg equilibrium in each of the three sampling periods (2003–2005, 2013, 2014) using the R package 'HardyWeinberg' (version 1.5.6, Graffelman 2015). We collected genetic data in 2013 and 2014, but not in 2012 or 2015. All statistical analyses were performed in R (version 3.2.5, R Core Team 2016).

Measuring selection

For the first 3 years of the study, we uniquely marked each individual lizard with manual injection visual implant elastomer (Northwest Marine Technology Inc.), which has been used effectively to identify reptiles in other capture–recapture studies (e.g. Penney *et al.* 2001). We used up to six different fluorescent colours in four locations on the ventral surface and upper thigh of the lizards. When elastomer tags were missing or faint, we

used a combination of photographs and ventral patterning to further help identify individuals.

To estimate survival probability, we used open population capture–recapture models (Pollock *et al.* 1990; Lebreton *et al.* 1992). Specifically, we employed Cormack–Jolly–Seber (CJS) models (Cormack 1964; Lebreton *et al.* 1992), which condition on the first capture of each individual in the data set and model recaptures only. The model provides estimates of the probability of detecting an individual during sampling (p) and the probability of surviving from time t to $t + 1$, (φ). Survival probability φ was logit-transformed and modelled as a function of covariates (Lebreton *et al.* 1992) including sex (binary; male or female), dorsal midline brightness (continuous) and number of derived *Mc1r* alleles. We used models that both interpreted number of derived alleles as a categorical covariate (i.e. genotype, with three categories) or as a continuous covariate (i.e. number of derived alleles). We modelled p as constant across time and individuals (also see next paragraph) and φ as constant across time. We estimated annual survival accounting for difference in time interval between subsequent sampling events.

We implemented the CJS model in a Bayesian framework, following Kéry & Schaub (2012) in part to help deal with missing data. For example, some individuals were missing information on phenotype (12 missing photographs) and genotype (the first 122 captured between 2011 and 2012 were not buccal swabbed). In the Bayesian framework, these missing data were treated as any other unknown quantity (e.g. model parameters) and estimated by formulating a model for the data. Dorsal midline brightness was approximately normally distributed among individuals, and we therefore modelled it as a normal random variable with mean μ and variance σ^2. We modelled missing genotype information as a categorical variable with three possible categories ($C = 0$, $Y = 1$ and $T = 2$ alleles) and cell probabilities p_0, p_1, and p_2. Parameters of the data models (i.e. μ and σ^2 for brightness, and p_0, p_1, and p_2 for genotype/number of alleles) are estimated as part of the model and are directly informed by observed individual covariate values. In all models, we assumed that individuals are equally likely to be detected independent of phenotype or genotype, that we are equally able to measure all covariates and that recorded individual covariate values are representative of the entire sampled population.

We implemented models in JAGS (Plummer 2003) version 4.2, using the package RJAGS version 4-6 (Plummer 2016) in R version 3.2.5 (R Core Team 2016). JAGS uses Markov chain Monte Carlo (MCMC) methods for parameter estimation, and for each model, we ran three parallel chains with 5000 iterations each, after discarding the 200 initial iterations as burn-in. We checked all chains for convergence using the R-hat statistic (Gelman & Hill 2006). R-hat values were <1.1 for all parameters in all models, indicating convergence. We present results as posterior mean, standard error and 95% Bayesian credible interval (BCI, Bayesian analog to 95% confidence interval).

Results

Relationship between genotype and phenotype

Contrary to expectations, *Mc1r* genotype in ecotonal *Sceloporus cowlesi* was not significantly correlated with dorsal brightness (ANOVA: $F_{2,152} = 2.36$, $P = 0.098$). We observed a trend towards brighter dorsal coloration in individuals homozygous for the derived *Mc1r* allele (Fig. 1). However, there were few ecotonal lizards homozygous or heterozygous for the derived *Mc1r* allele ($N = 11$ and $N = 20$, respectively) relative to individuals homozygous for the wild-type allele ($N = 124$), reducing the power to draw definitive conclusions about the relationship between genotype and phenotype. Regardless, our results seem to contrast with previous observations for a combined sample of White Sands, ecotone and dark soils *S. cowlesi* (Rosenblum *et al.* 2010). When samples from all habitats were evaluated together in a previous study, individuals homozygous for the derived *Mc1r* allele were significantly brighter in dorsal coloration than individuals

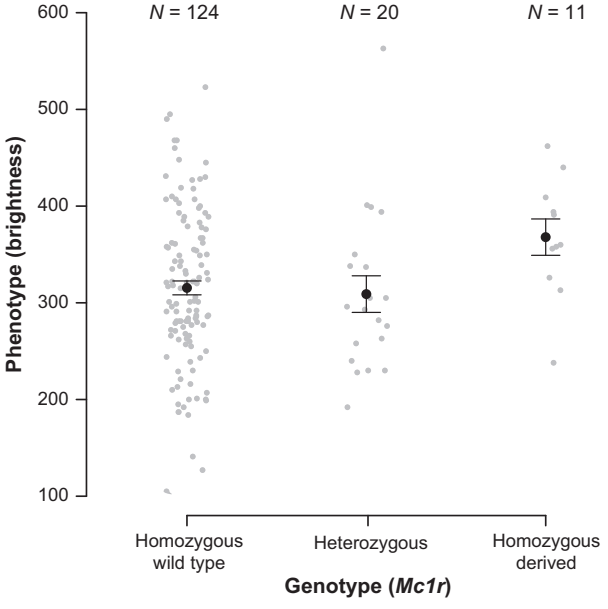

Fig. 1 Variation in dorsal midline brightness with standard error bars for *Sceloporus cowlesi* by *Mc1r* genotype at the White Sands ecotone.

homozygous for the wild-type allele and heterozygotes were statistically indistinguishable in colour from homozygotes for the derived allele (Rosenblum *et al.* 2010). However, if we reanalyse the small number of ecotonal samples ($N = 14$) from Rosenblum *et al.* 2010, the results are concordant: there are no significant differences in dorsal colour across genotypic classes for ecotonal *S. cowlesi* (ANOVA: $F_{2,11} = 0.22$, $P = 0.80$). Thus, both previous and current results indicate that the strong pattern of association between *Mc1r* genotype and colour phenotype appears to be driven by samples from the heart of the dunes, not by samples from the ecotone.

Allele frequencies over time

We found that the frequency of the derived *Mc1r* allele has decreased over time in the *S. cowlesi* sampled at the White Sands ecotone. The frequency of the derived allele was 0.46 in ecotonal samples collected in 2003–2005 (Rosenblum *et al.* 2010), 0.16 in 2013 and 0.13 in 2014 (Fig. 2, left). Despite differences in sample sizes (i.e. a smaller sample from 2003 to 2005), the proportion of derived alleles differed significantly across sampling periods (Fisher's Exact Test: $P = 0.0002$); however, allele frequencies were not significantly different between 2013 and 2014 when tested independently (Fisher's exact test: $P > 0.05$). The decrease in overall frequency of the derived *Mc1r* allele at the ecotone was due to a decrease in both genotypic classes that carry the derived allele. Specifically, between the 2003–2005 and 2013 sampling points, there was a 68% decrease in heterozygotes and a 70% decrease in homozygotes for the derived allele (Fig. 2, right). From 2013 to 2014, there was a further 24% decrease in heterozygotes and a further 5% decrease in homozygotes for the derived allele (Fig. 2, right). Unlike *Mc1r* data collected from the 2003–2005 samples, which did not deviate significantly from Hardy–Weinberg equilibrium ($\chi^2 = 0.16$, $P > 0.05$), *Mc1r* data collected from the 2013 and 2014 samples deviated significantly (2013: $\chi^2 = 5.34$, $P < 0.05$; 2014: $\chi^2 = 17.68$, $P \ll 0.0001$).

Selection on genotype and phenotype

We captured a total of 276 individuals with approximately equal proportions of males and females (146 and 132, respectively, $\chi^2 = 0.7$, $P = 0.4$) over 4 years. Most individuals (203) were only captured once; 63 were captured twice; 10 were captured three times; two were captured four times; and only one individual was captured during all five sampling periods. Our data suggest that *S. cowlesi* can sometimes live longer than 4 years, which is higher than previously reported lifespans for closely related species in the wild (e.g. *Sceloporus undulatus*: 1.75–3.83 years, Tinkle & Ballinger 1972). All models provided consistent estimates of average annual survival (approximately 0.4) and detection probability (approximately 0.5).

Mc1r genotype, but not colour phenotype, appeared to have an effect on survival probability of *S. cowlesi* on the ecotone. Unexpectedly, the number of derived *Mc1r* alleles had a negative effect on survival probability ($\beta = -0.296$, SE = 0.222; Table 1). Even though 95% BCI overlapped 0, most of the posterior probability was concentrated below 0, providing evidence of a considerable decrease in survival for individuals carrying the derived allele (Fig. 3 top, Table 1). Models using genotype as a categorical covariate gave very similar estimates of genotype-specific survival, indicating that modelling the number of alleles as a logit linear predictor of survival was adequate (Table 1). Model results indicated that effects of dorsal brightness and of sex on

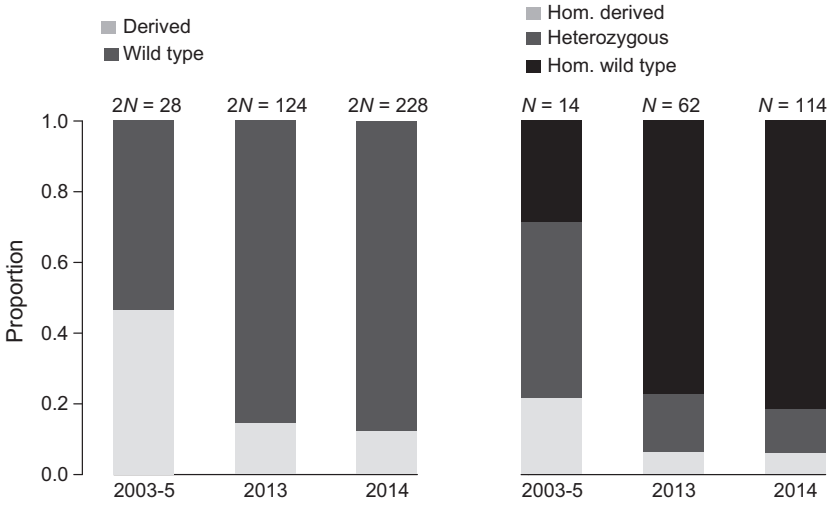

Fig. 2 Changes over time in proportion of derived *Mc1r* allele (left) and proportion of individuals sampled that were homozygous for the derived allele, heterozygous and homozygous for the wild-type allele (right) in ecotonal *Sceloporus cowlesi* over an approximately 10-year period. Sampling areas for the two time points overlapped, but samples in 2003–2005 were taken from more dispersed ecotonal localities, whereas those taken in 2013 and 2014 were taken from a single locality.

Table 1 Parameter estimates from Cormack–Jolly–Seber model applied to 4-year capture–recapture data of *Sceloporus cowlesi* surveyed in the White Sands ecotone from 2012 to 2015. The model includes a logit linear effect of number of alleles (β) on survival. The table shows the posterior mean, standard deviation and lower (2.5%) and upper (97.5%) limits of 95% Bayesian credible interval where p = detection probability, ϕ = annual survival probability (based on 0, 1 and 2 alleles) and p_0, p_1, p_2 = probability of having 0, 1, or 2 alleles, respectively.

Parameter	Mean	SD	2.5%	97.5%
p	0.488	0.067	0.362	0.623
p_0	0.786	0.034	0.717	0.848
p_1	0.135	0.027	0.086	0.192
p_2	0.079	0.022	0.042	0.128
ϕ_0	0.423	0.059	0.318	0.548
ϕ_1	0.32	0.076	0.189	0.486
ϕ_2	0.232	0.123	0.052	0.525
β	−0.296	0.222	−0.702	0.172

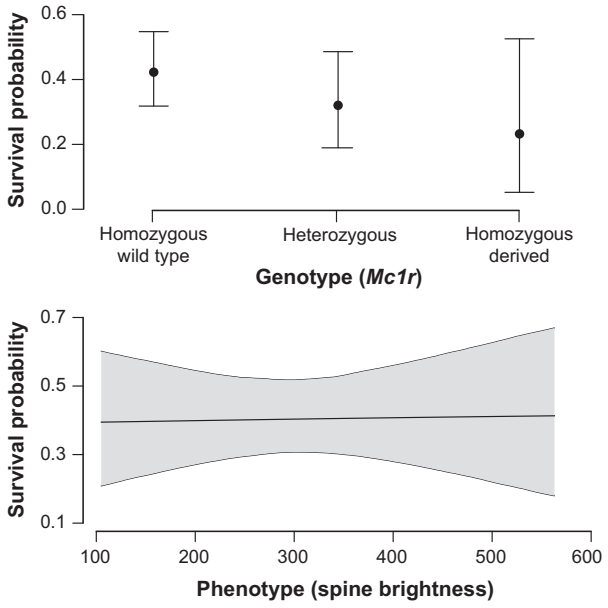

Fig. 3 Capture–recapture estimates of survival probability (with 95% Bayesian confidence intervals) as a function of *Mc1r* genotype (top) and spine brightness phenotype (bottom) of *Sceloporus cowlesi* at the White Sands ecotone.

survival were negligible (on the logit scale, β_{sex}: 0.027, SE = 0.213; $\beta_{brightness}$: 0.010, SE = 0.106).

Discussion

Evolutionary biologists are often inspired by adaptation in the wild. The seeming fit between organisms and their environments – whether in functional traits (e.g. beak morphology of Galapagos finches, Grant & Grant 1989), appearance (e.g. cryptic colour in peppered moths, Kettlewell 1959, 1961) or behaviour (e.g. antipredator strategies in Trinidadian guppies, O'Steen *et al.* 2002) – often motivates research on the mechanisms of selection in the wild. However, it is not uncommon for researchers to discover that dynamics of natural selection are far more complex and nuanced than initially assumed. For example, studies in natural systems over longer time scales often show that the strength, mode or direction of selection can be variable over time (e.g. beak size and shape in Galapagos finches, Grant & Grant 1989) and that understanding mechanisms of selection can be less straightforward than expected (e.g. avian predation in peppered moths, Howlett & Majerus 1987). Further, studies on the genetic basis of adaptation often reveal complex dynamics including the importance of plasticity (e.g. *Anolis* limb length, Losos *et al.* 2000), pleiotropy (e.g. *Catsup* affecting behavioural, physiological and morphological traits in *Drosophila melanogaster*, Carbone *et al.* 2006) and polygenetic effects (e.g. flowering time in *Arabadopsis thaliana*, Brachi *et al.* 2010).

Our analysis of capture–recapture data for *Sceloporus cowlesi* at White Sands provides another example of how studies of ongoing natural selection in the wild can reveal unexpected patterns. We integrated genotype, phenotype and survival data to understand dynamics of natural selection on *S. cowlesi* at the White Sands ecotone. Our study revealed several unanticipated findings about the relationship between *Mc1r* genotype and colour phenotype, changes in *Mc1r* allele frequencies over time and patterns of survival in ecotonal *S. cowlesi*, as detailed below.

Based on our previous work, we expected to find a strong association between *Mc1r* genotype and dorsal colour phenotype. Prior research demonstrated a strong association between a particular amino acid substitution in *Mc1r* and blanched coloration in *S. cowlesi* (Rosenblum *et al.* 2004, 2010). Functional assays confirmed that the mutation has functional consequences (Rosenblum *et al.* 2010). Moreover, there is a strong molecular signature of natural selection at *Mc1r* and the surrounding gene region (Laurent *et al.* 2016). However, ecotonal *S. cowlesi* samples in the current study showed minimal association between dorsal midline brightness and *Mc1r* genotype. Individuals homozygous for the derived *Mc1r* allele tended to be brighter, but this trend was not statistically significant. Thus, our results initially seemed to contrast with previous observations. However, prior studies that showed a strong association between *Mc1r* genotype and colour phenotype considered samples from all habitats combined (i.e. White Sands, ecotone, and dark soils, Rosenblum *et al.* 2010).

Instead, the striking pattern of association between *Mc1r* genotype and blanched coloration was driven by samples from the heart of the White Sands formation.

Another unexpected result of our study was the apparent decrease in the frequency of the derived *Mc1r* allele in ecotonal *S. cowlesi* over the last 10 years. Samples taken from the White Sands ecotone in 2003–2005 showed that the derived *Mc1r* allele was at a frequency of nearly 0.5. Samples for the current study, taken 10 years later, show a frequency of the derived *Mc1r* allele at <0.2. We observed the decline of the derived *Mc1r* allele in both heterozygous and homozygous genotypic classes. Furthermore, *Mc1r* data from the 2003 to 2005 samples were consistent with Hardy–Weinberg expectations, but the *Mc1r* data from 2013 to 2014 deviated significantly from equilibrium. There are numerous factors that could potentially contribute to deviations from Hardy–Weinberg equilibrium in this system including assortative mating (Hardwick *et al.* 2013), gene flow from the surrounding dark soils populations (e.g. Rosenblum *et al.* 2007; Laurent *et al.* 2016) and natural selection (as discussed below). It is important to note that the 2003–2005 sample size was much smaller than our 2013–2014 sample size (14 vs. 157 unique ecotonal individuals) and was taken from more dispersed sampling localities, so conclusions should be made with caution. The 2013–2014 samples were all collected from a single locality of approximately 10-ha area, while the 2003–2005 samples were taken from a larger 12-km stretch across the southeast part of the ecotone. However, the sampling areas for the two time points overlapped, and the derived *Mc1r* allele frequency continued to decrease slightly during the current study (2013–2014) in the same locality.

Consistent with the observation that the derived *Mc1r* allele declined over the last 10 years, our capture–recapture data revealed a lower survival rate for *S. cowlesi* individuals carrying the derived *Mc1r* allele. Specifically, individuals homozygous for the wild-type *Mc1r* allele indicated a higher annual survival rate (over 40%) than individuals carrying a derived allele. Thus, our results imply that selection at the ecotone may have favoured the wild-type allele in recent years, thus providing a mechanism for its observed increase in frequency. Also our capture–recapture results do not indicate differential survival of individuals based on dorsal brightness, which is again consistent with our data showing only weak association with *Mc1r* genotype and colour phenotype for ecotonal *S. cowlesi*.

Given multiple lines of evidence that a particular *Mc1r* mutation contributes to blanched coloration in White Sands *S. cowlesi*, why would patterns be different at the ecotone? Moreover, what could account for decreased survivorship of ecotonal lizards carrying the derived *Mc1r* allele? Our unexpected findings suggest the importance of continued research in several key areas, particularly those that integrate organismal, molecular and genomic approaches.

First, our results highlight the need to understand dynamics of phenotypic plasticity at the ecotone. Previous common-garden rearing and physiological plasticity studies demonstrated that differences in coloration between White Sands and dark soils *S. cowlesi* could not be explained by phenotypic plasticity (Rosenblum 2006). However, these studies compared lizards from the heart of White Sands to lizards from dark soils and did not include ecotonal animals. We do not yet know whether ecotonal individuals exhibit greater phenotypic lability than those in the heart of the dunes, and further work will be important to assess phenotypic plasticity and its role for individual fitness in ecotonal *S. cowlesi*.

Second, it is important to document what genes besides *Mc1r* contribute to colour variation in *S. cowlesi*. *Mc1r* does not explain all of the variation in dorsal coloration in this system, even for lizards in the heart of the dunes. Thus, other genetic factors must interact with *Mc1r* to affect colour phenotype. There are a number of other genes important in the vertebrate melanin synthesis pathway (e.g. Gross *et al.* 2009; Manceau *et al.* 2010), and candidate gene studies and whole genome comparisons will likely be fruitful for obtaining a more complete picture of the genetic architecture of blanched coloration in this system. Given high levels of colour variation at the ecotone and ongoing gene flow between White Sands and dark soil *S. cowlesi* (Rosenblum 2006; Rosenblum *et al.* 2007; Rosenblum & Harmon 2011; Laurent *et al.* 2016), the ecotone population may in fact provide an outstanding opportunity for using admixture mapping approaches to add to our understanding of the genetic basis of colour variation.

Third, increased survival of individuals with the wild-type *Mc1r* allele at the ecotone suggests the value of exploring possible pleiotropic effects of *Mc1r* in the White Sands system. In natural vertebrate populations, mutations in *Mc1r* have received the most attention for their effect on coloration [e.g. fish (Gross *et al.* 2009), amphibians (Matsuba 2012), reptiles (Rosenblum *et al.* 2004), birds (Mundy *et al.* 2005) and mammals (Nachman *et al.* 2003; Pérez *et al.* 2013)]. However, *Mc1r* may have pleiotropic effects, particularly for behavioural and physiological traits (Ducrest *et al.* 2008). In particular, darker individuals may be more aggressive, stress-resistant and sexually active than their lighter counterparts (see review in Ducrest *et al.* 2008). Selection may especially favour more aggressive individuals when intraspecific competition is high (e.g. Stamps 1977). Previous studies on *S. cowlesi* showed behavioural differences between dark soils and White Sands individuals

(Robertson & Rosenblum 2010; Robertson *et al.* 2011; Hardwick *et al.* 2013; Des Roches *et al.* 2014); however, these studies did not assess ecotonal lizards nor genotype *Mc1r*. Thus, understanding whether variation at *Mc1r* may correlate with phenotypic variation besides colour is particularly interesting for populations at the White Sands ecotone, where population density – and likely intraspecific competition – is particularly high.

Fourth, it is possible that changes in the balance between selection and gene flow contribute to our observed patterns. Declines in the numbers of visual predators – for example, the American Kestrel and Loggerhead Shrike (Sauer *et al.* 2013) – may lead to relaxed selection pressures. Moreover, selection for crypsis in *S. cowlesi* is likely already weaker at the White Sands ecotone than in the heart of the dunes. Ecotone populations of *S. cowlesi* are primarily associated with vegetation rather than exposed sand substrate (Refsnider *et al.* 2015). If selection has indeed been relaxed on ecotonal *S. cowlesi* (whether for dorsal coloration or other traits), dynamics of gene flow would likely lead to an increase in the wild-type *Mc1r* allele. Previous research has documented ongoing gene flow between White Sands and dark soil *S. cowlesi* (Rosenblum 2006; Rosenblum *et al.* 2007; Rosenblum & Harmon 2011; Laurent *et al.* 2016), and the wild-type *Mc1r* allele is at (or near) fixation in nearby dark soils populations (Rosenblum *et al.* 2010). Genotyping additional historical and contemporary samples from different localities along the ecotone, continuing to monitor *Mc1r* allele frequencies at the same location in the future, assessing patterns of gene flow across finer spatial scales and comparing patterns observed at the ecotone with those in the heart of the dunes will all be important for understanding shifting dynamics of selection and gene flow at White Sands.

Ultimately, integrating molecular, genomic and organismal approaches will be essential for disentangling the relative contribution of different factors to observed patterns. At White Sands, organismal approaches have been crucial for documenting patterns of phenotypic variation (e.g. Rosenblum & Harmon 2011; Des Roches *et al.* 2014, 2015a,b) and beginning to understand their fitness consequences (e.g. this study and Hardwick *et al.* 2015). Molecular approaches have been effective for identifying candidate genes that contribute to colour variation and understanding the functional consequences of specific mutations (e.g. Rosenblum *et al.* 2010). Genomic approaches have been important for understanding the demographic backdrop for selection and for identifying signatures of natural selection at the genome level (e.g. Laurent *et al.* 2016). Moving forward, these approaches can be explicitly integrated by simultaneously measuring survival, tracking specific mutations known to have phenotypic consequences and conducting genomic scans for additional loci under selection.

Our research on differential survivorship of *S. cowlesi* on the White Sands ecotone is noteworthy for concurrently measuring selection on both genotype and phenotype using a capture–recapture approach. Our results are unanticipated, in particular because they are consistent with selection *against* the derived *Mc1r* genotype, which has been shown to contribute to cryptic blanched coloration in this species. Our findings suggest that evaluating natural selection on genotype and phenotype simultaneously can reveal unexpected patterns. Even in a system where a genotype–phenotype association appeared to be black and white, our study suggests that additional factors – including phenotypic plasticity, epistasis, pleiotropy and gene flow – may play important roles at the ecotone. More generally, our findings indicate that patterns of natural selection can be especially complex in transitional habitats like ecotones and point to the value of future research examining dynamics of ongoing selection in other ecological transition zones.

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Data accessibility

All data (capture–recapture history, Mc1r genotype and colour phenotype for each individual) are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.f79n4.