

# Molecular and functional basis of phenotypic convergence in white lizards at White Sands

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There are many striking examples of phenotypic convergence in nature, in some cases associated with changes in the same genes. But even mutations in the same gene may have different biochemical properties and thus different evolutionary consequences. Here we dissect the molecular mechanism of convergent evolution in three lizard species with blanched coloration on the gypsum dunes of White Sands, New Mexico. These White Sands forms have rapidly evolved cryptic coloration in the last few thousand years, presumably to avoid predation. We use cell-based assays to demonstrate that independent mutations in the same gene underlie the convergent blanched phenotypes in two of the three species. Although the same gene contributes to light phenotypes in these White Sands populations, the specific molecular mechanisms leading to reduced melanin production are different. In one case, mutations affect receptor signaling and in the other, the ability of the receptor to integrate into the melanocyte membrane. These functional differences have important ramifications at the organismal level. Derived alleles in the two species show opposite dominance patterns, which in turn affect their visibility to selection and the spatial distribution of alleles across habitats. Our results demonstrate that even when the same gene is responsible for phenotypic convergence, differences in molecular mechanism can have dramatic consequences on trait expression and ultimately the adaptive trajectory.

adaptation | genetics | lizard | *Mc1r* | speciation

Convergent evolution of similar phenotypes in similar environments has long been taken as evidence of adaptation driven by natural selection (1, 2). An outstanding question, however, is whether such convergence results from similar or different mechanisms. There are multiple levels at which mechanistic causes of convergence can occur. Are changes in the same developmental or physiological pathways implicated in phenotypic convergence? If so, are changes at the same specific gene(s) responsible for convergent phenotypes? And, finally, if the same genes are employed, are the underlying molecular and functional mechanisms linking genotype to phenotype conserved? Whereas several studies have shown that the same pathway or the same gene is responsible for convergence (3), few studies have tested the functional effects of evolutionarily independent mutations within the same gene to determine whether they are equivalent. Different mutations in the same gene can act through unique functional mechanisms even if they result in similar phenotypic effects—alleles may differ in their dominance and net selection coefficients (e.g., due to differences in pleiotropy and/or epistatic interactions), which can affect the probability and rate of fixation of alleles in the wild. Thus, understanding the functional basis of adaptation has far-reaching implications, as it sheds light on the predictability of mechanisms generating adaptive change.

Here we test whether independent mutations in the melanocortin-1 receptor (*Mc1r*) gene have similar functional consequences in three lizard species with convergent adaptations to a common novel environment. The White Sands formation in the Chihuahuan Desert is a stark landscape of white gypsum dunes. Formed less than 6000 years ago (4), these sand dunes represent a dramatic new selective environment for small diurnal animals

subject to visual predation. Three species of lizards [Eastern Fence Lizard (*Sceloporus undulatus*), Little Striped Whiptail (*Aspidoscelis inornata*), and Lesser Earless Lizard (*Holbrookia maculata*)] have each evolved blanched, substrate-matched phenotypes at White Sands (Fig. 1A). The convergent phenotypes have evolved rapidly under strong divergent selection from a brown, ancestral phenotype found on the dark adobe soils in the surrounding desert (5).

Previously, we identified an association between adaptive pigmentation and mutations for all three taxa in *Mc1r*, a gene that plays a critical role in the production of vertebrate melanin pigments (6). In each species, a single derived amino acid replacement (different in each species) was statistically associated with the blanched coloration of White Sands lizards (Fig. 1B). Here we use functional assays to connect the mechanism of disruption of the *Mc1*-receptor to patterns of allelic dominance and the distribution of alleles in natural habitats.

## Results and Discussion

Using a large sample of lizards from white sand and dark soil habitats, we confirm that the statistical association between a single *Mc1r* mutation in each species and color was highly significant for each species (*S. undulatus*:  $n = 114$ ,  $P < 10^{-9}$ ; *A. inornata*:  $n = 100$ ,  $P < 10^{-15}$ ; *H. maculata*:  $n = 88$ ,  $P < 10^{-24}$ , Table S1). The genotype-phenotype association was not always perfect (some ancestral alleles were found in white sand populations of both *S. undulatus* and *A. inornata*), suggesting that additional genes also contribute to color variation. All three mutations occur in transmembrane (TM) regions, which are important for maintaining the structural integrity of the receptor and play a known role in both ligand binding and signaling (7). In *S. undulatus* the replacement from histidine (wild-type) to tyrosine (derived) at amino acid residue 208 (His<sup>208</sup>Tyr) is a change from a positively charged to an aromatic, uncharged amino acid. Histidine is conserved at this residue across vertebrates, suggesting it is important for *Mc1r* function. In *A. inornata* the Thr<sup>170</sup>Ile replacement is a polarity-changing replacement at a position that is also implicated in mutationally induced changes of *Mc1*-receptor function in humans (8). Unlike the other two mutations, in *H. maculata* the Val<sup>168</sup>Ile replacement is perfectly associated with color variation, but is a conservative change between physiochemically similar amino acids, both of which are common in other species (e.g., human *Mc1r*, Ile<sup>168</sup>) and *Mc1r* paralogs (e.g., human *Mc4r*, Ile<sup>173</sup>).

To investigate the functional consequences of these *Mc1r* mutations, we expressed wild-type and derived *Mc1r* alleles from all three lizard species and measured receptor signaling. Specifi-

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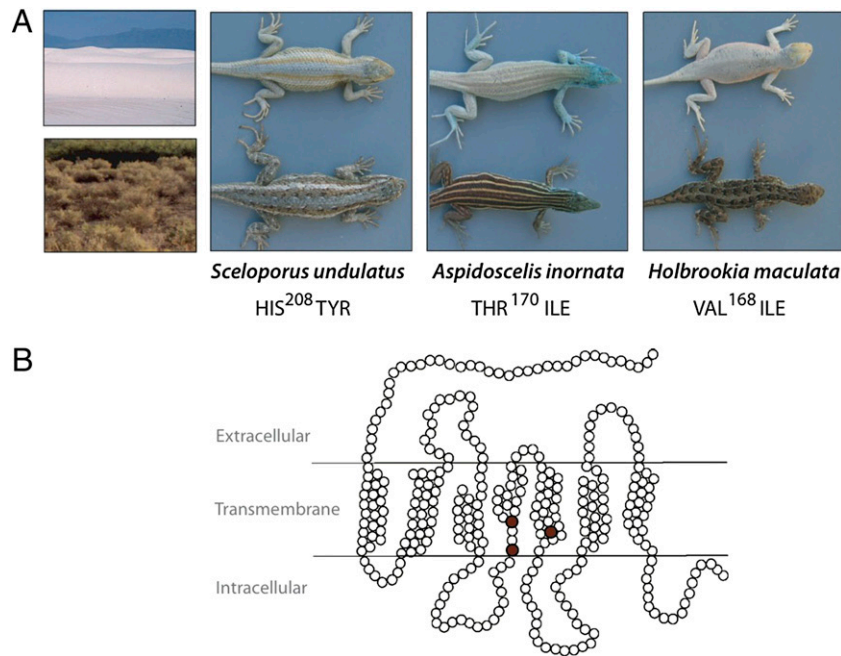
The authors declare no conflict of interest.

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**Fig. 1.** Mutations associated with blanced coloration in White Sands lizards. (A) Blanced morphs from white sands on top and dark morphs in ancestral dark soil habitat on bottom. (B) Amino acid schematic of the melanocortin-1 receptor (Mc1r); replacements statistically associated with coloration in the focal taxa are shown in red.

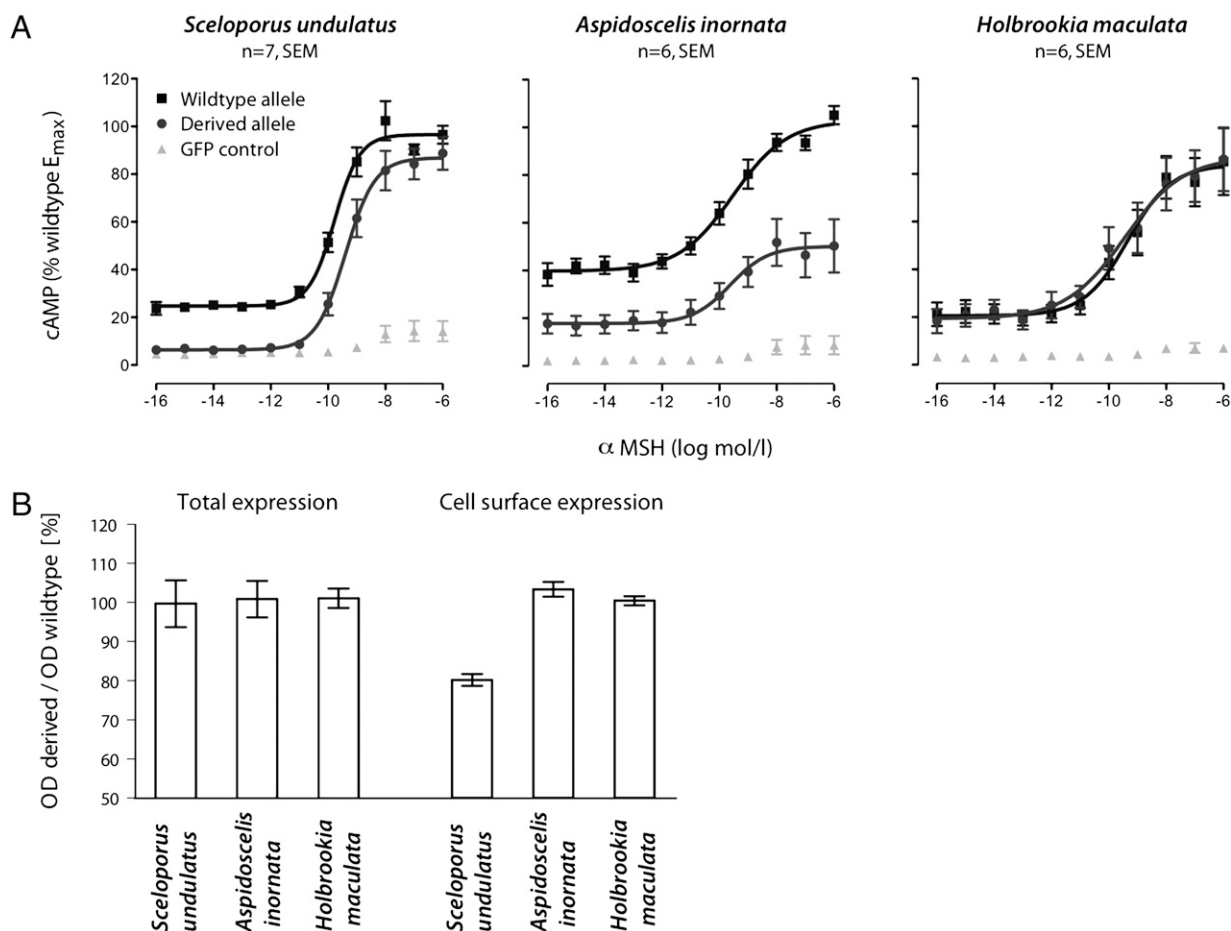
cally, we performed heterologous cell-based assays of signal transduction efficiency for all six *Mc1r* alleles in mammalian COS-7 cells by measuring agonist-induced intracellular cyclic adenosine monophosphate (cAMP) levels (Fig. 2A; Table S2). All alleles responded to the natural agonist  $\alpha$ -MSH [which is highly conserved across vertebrates (9)] with an increase in intracellular cAMP levels. However, for *S. undulatus* and *A. inornata*, the derived alleles exhibited reduced basal and agonist-induced cAMP formation relative to their respective wild-type alleles. The  $^{208}\text{Tyr}$  allele from blanced *S. undulatus* showed a 24% reduction in agonist-induced cAMP formation and a 68% decrease in basal cAMP formation (paired *t* test,  $P < 0.05$ ) compared to the wild-type allele. Similarly, the  $^{170}\text{Ile}$  allele from blanced *A. inornata* showed a 62% reduction in agonist-induced cAMP formation (paired *t* test,  $P < 0.05$ ) and a 62% decrease in basal cAMP formation (paired *t* test,  $P < 0.05$ ). Reductions in receptor signaling of similar magnitude have pronounced effects on pigmentation and are associated with light-colored hair in mice and humans (10, 11). Thus, this assay demonstrates repeated use of the same gene in adaptive evolution in phylogenetically distant species. Both *S. undulatus* and *A. inornata* [which share a most recent common ancestor  $\approx 175$  million years ago (12)] evolved blanced phenotypes via partial loss-of-function *Mc1r* mutations.

Although *H. maculata* showed the strongest statistical association between *Mc1r* mutation (Val $^{168}\text{Ile}$ ) and color, our functional assays showed no measurable differences in agonist-induced cAMP levels. Thus, the Val $^{168}\text{Ile}$  mutation may be (i) simply a spurious statistical association, (ii) in linkage disequilibrium with a functionally relevant noncoding mutation, or (iii) affecting *Mc1r* function in a way that we did not measure (e.g., mRNA stability). In any case, it is clear that *H. maculata* has evolved coloration via a different mechanism than the other two species and underscores the importance of functional tests in evolutionary studies (13).

The reduction in basal and agonist-induced cAMP levels in derived *S. undulatus* and *A. inornata* *Mc1r* alleles suggested two possible functional mechanisms: lower cell-surface expression levels and/or a reduced coupling efficiency of Mc1r. To discriminate

between these two possibilities, we quantified cell-surface expression levels of wild-type and derived alleles. Specifically, we measured *Mc1r* protein-expression levels in all cellular compartments and at the plasma membrane using a total cellular ELISA and a cell-surface ELISA, respectively. Total receptor protein expression did not differ between the wild-type and derived alleles in transiently transfected cells in either species; however, cell-surface expression level of the *S. undulatus*  $^{208}\text{Tyr}$  allele was reduced by 20% (paired *t* tests,  $P < 0.0001$ ; Fig. 2B; Table S2). This reduction in cell-surface expression suggests a partial intracellular retention of this protein similar to that observed for human *Mc1r* alleles associated with red hair (14). In other words, the reduced activity of the derived *S. undulatus* receptor is caused by a reduction in its ability to integrate into the melanocyte membrane efficiently (i.e., trafficking deficiency). In contrast, the dysfunction of the *A. inornata* derived allele cannot be attributed to a difference in the receptor's cell-surface expression. Instead, the reduced activity of the  $^{170}\text{Ile}$  allele is likely due to a change in its ability to transduce signals (e.g., impaired G-protein-coupling efficacy), as is the mechanism for well-characterized *Mc1r* mutations in humans (e.g., ref. 11). Therefore, the independent *Mc1r* replacements produce similar phenotypic results via entirely different functional mechanisms.

These differences in the molecular underpinnings of *Mc1r* disruption lead to important organismal-level consequences. We evaluated the effect of *Mc1r* genotype on dorsal coloration for samples of both species (Table S3) and found significant differences in color among genotypic classes [*S. undulatus*:  $F_{(2,49)} = 42.15$ ,  $P < 10^{-5}$ ; *A. inornata*:  $F_{(2,36)} = 15.00$ ,  $P < 10^{-5}$ ]. In both species, individuals homozygous for the derived *Mc1r* allele were significantly lighter in color than individuals homozygous for the wild-type allele. However, for *S. undulatus*, heterozygotes were statistically indistinguishable in color from individuals homozygous for the derived allele, whereas for *A. inornata*, heterozygotes were statistically indistinguishable in color from individuals homozygous for the wild-type allele (Fig. 3). Thus, we can determine dominance patterns for *Mc1r* alleles in the focal taxa, even though controlled breeding studies cannot be conducted for these species. The derived *Mc1r* allele appears to be

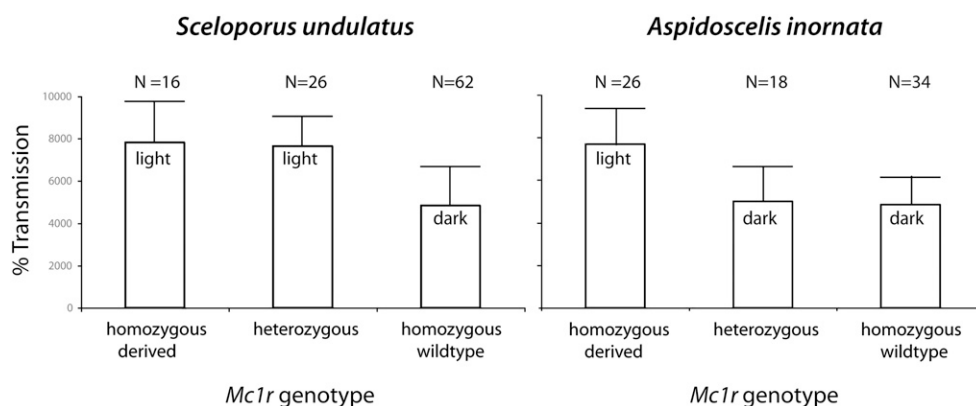


**Fig. 2.** Functional assays for *Mc1r* in White Sands lizards. (A) Cell-based assays identifying partial loss-of-function *Mc1r* alleles for blancher *S. undulatus* and *A. inornata*. Derived and wild-type alleles in a mammalian expression vector were tested for basal and agonist-induced cAMP accumulation in response to increasing concentrations of  $\alpha$ -MSH. Green fluorescent protein (GFP) plasmid-transfected cells served as controls. (B) ELISAs identifying reduced cell-surface expression for the derived *S. undulatus* *Mc1r* allele. Specific optical density (OD) readings (OD value of HA-tagged construct minus OD value of control-transfected cells) are given for each species as a percentage of the wild-type variant for total and cell-surface expression.

dominant in *S. undulatus* but recessive in *A. inornata*. Therefore, mutations in the same gene associated with similar phenotypes differ in dominance.

This difference in genetic dominance is consistent with what is known about *Mc1r* function. Mutations causing a reduction in

membrane integration efficiency (as demonstrated in *S. undulatus*) are expected to be dominant. Because G-protein-coupled receptors (GPCRs) dimerize (15), if wild-type and mutant receptors are coexpressed (in a heterozygote), the mutant receptor can retain the wild-type receptor in the cellular interior and prevent



**Fig. 3.** Dominance relationships of *Mc1r* alleles. Dorsal coloration (mean and standard deviation for area under the spectral curve) for *Mc1r* genotypes showing the derived allele is dominant in *S. undulatus* and recessive in *A. inornata*. *n*, number of alleles sampled; "light" and "dark" refer to statistically distinguishable groups.

its integration in the membrane. This mechanism is one explanation for the many examples of dominant reduced-function mutations in *Mc1r* paralogs (16) and other GPCRs (17). In contrast, mutations causing a disruption of receptor signaling (as observed for *A. inornata*) are expected to be recessive, and recessive *Mc1r* signaling mutations are found to cause lighter coloration in other taxa (18, 19).

The observed differences in dominance also affect the spatial patterns of allelic variation in the wild. We measured *Mc1r* allele frequencies in three habitats: white sand (center of the gypsum dunes), dark soil (surrounding desert grasslands), and the ecotone (edge of the dune field with more variable, intermediate substrate). The distribution of *Mc1r* alleles across habitats differed between the taxa (Fig. 4). For example, nearly all light-colored *A. inornata* in white sand habitat were homozygous for the derived (recessive) *Mc1r* allele, whereas many light-colored *S. undulatus* in white sand habitat (in which the derived *Mc1r* allele is dominant) were heterozygous. The difference in the distribution of *Mc1r* alleles across habitat types can be largely explained by allelic differences in dominance; that is, selection will efficiently remove the visible dominant allele in the mismatched habitat. Moreover, because the distribution of *Mc1r* allelic variation is consistent with expectations based on the distribution of phenotypes, this provides strong evidence that color phenotype is, in fact, the direct target of selection (rather than a pleiotropic effect of another selection target).

Here we report a natural system of multiple species under an identical selection regime using the same gene but different molecular mechanisms to generate convergent phenotypes. We suspect that convergence through different mutations with different functional effects may be common for partial loss-of-function (as opposed to gain-of-function) mutations because there are likely to be many ways to “break” a pathway or disrupt gene function. Importantly, we show that differences in functional mechanisms of mutations can affect color phenotype, allelic dominance, and the geographic distribution of alleles in nature. These differences in the genetic architecture of locally adapted populations can influence rates of adaptation and gene flow, which are also critical parameters in predicting the likelihood and rate of parapatric speciation (20). Further, in addition to changes in concealing coloration, white sand and dark soil populations exhibit population differences in color patches used for social signaling (21). The observed *Mc1r* mutations and their consequences for receptor function are therefore associated with convergent phenotypes important for both adaptation and speciation in recently diverged lizards in the wild.

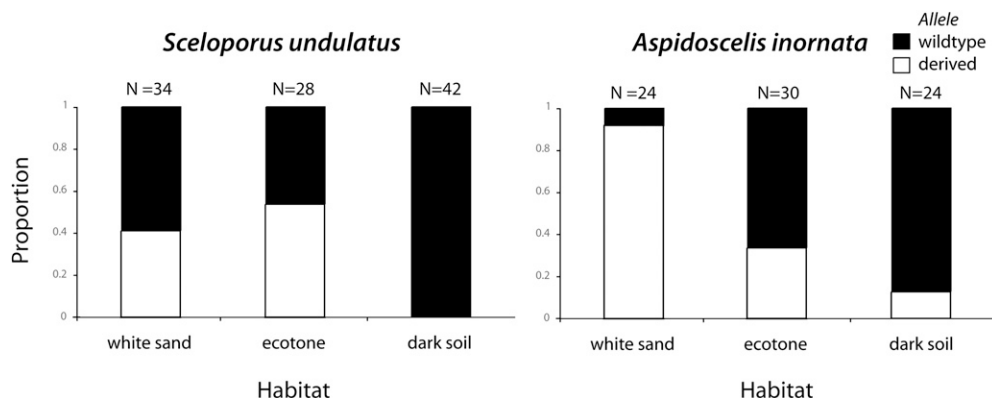
## Materials and Methods

**Determining the Functional Effects of *Mc1r* Mutations. Functional assays.** To determine the functional consequences of the amino acid replacements observed in *Mc1r* for White Sands lizards, we inserted full-length reptile *Mc1r* cDNA from dark soil individuals with wild-type *Mc1r* alleles (*Sceloporus undulatus* AY586117.1, *Aspidoscelis inornata* AY586036.1, *Holbrookia maculata* AY586075.1) into the mammalian expression vector pcDps and epitope-tagged with an N-terminal hemagglutinin (HA) and a C-terminal Flag epitope by PCR mutagenesis. We then introduced *Mc1r* mutations associated with the derived allele in White Sands individuals (*S. undulatus* AY586146.1, *A. inornata* AY586051.1, *H. maculata* AY586091.1) into the tagged wild-type *Mc1r* constructs using a PCR-based site-directed mutagenesis and restriction fragment replacement strategy. We confirmed the accuracy of all PCR-derived sequences by restriction analysis and sequencing.

**Cell culture and transfection.** We cultivated COS-7 cells in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified 7% CO<sub>2</sub> incubator. For cell transfection, we used Lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction.

**ALPHAScreen cAMP assay.** Because MC1R mediates its signal via G<sub>s</sub>/adenylyl cyclase activation, we measured agonist-induced intracellular cAMP levels. The cAMP content of cell extracts was determined by a nonradioactive cAMP assay based on the ALPHAScreen technology (Perkin-Elmer, ref 22). Thus, we split cells into 50-mL cell-culture flasks (1 × 10<sup>6</sup> cells/flask) and transfected them with 5 µg of plasmid DNA. One day after transfection, we seeded cells into 48-well plates (5 × 10<sup>4</sup> cells/well). One day later, we performed cAMP accumulation assays. Cells were washed once and incubated in serum-free DMEM containing 1 mM 3-isobutyl-1-methylxanthine (Sigma) in the absence or in increasing amounts of agonist (α-MSH; Sigma) for 1 h at 37°C. We terminated the reactions by aspirating media and lysed cells in 50 µL lysis buffer (see ALPHAScreen manual) containing 1 mM 3-isobutyl-1-methylxanthine. From each well, we transferred 5 µL lysate into a 384-well plate and added acceptor/donor beads according to the manufacturer’s protocol. We analyzed the data from the ALPHAScreen cAMP assay using the GraphPad Prism program (version 5.01 for Windows).

**ELISAs.** To ligand-independently estimate the amounts of receptor proteins expressed at the cell surface and in all cellular compartments, we used an indirect cell-surface ELISA and a total cellular ELISA, respectively (23). In brief, to estimate cell-surface expression of receptors carrying an amino-terminal HA tag, transfected COS-7 cells were seeded into 48-well plates 24 h after transfection. Cells were fixed with 4% paraformaldehyde without disrupting the cell membrane 72 h after transfection, and incubated with a peroxidase-coupled monoclonal anti-HA antibody (3F10; Roche). We then detected bound anti-HA antibody by adding H<sub>2</sub>O<sub>2</sub> and o-phenylenediamine (2.5 mM each in 0.1 M phosphate-citrate buffer, pH 5.0) as substrate and chromogen, respectively. After 15 min at room temperature, we terminated the enzyme reaction by adding 1 M HCl containing 0.05 M Na<sub>2</sub>SO<sub>3</sub>, and measured color development bichromatically at 492 and 620 nm using an ELISA reader (Sunrise; Tecan). For detection of total cellular expression, we harvested COS-7 cells 3 days after transfection (4 µg of plasmid DNA/60-mm dish), added 150 µL solubilization buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% deoxycholate, 1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, and 10 µg/mL aprotinin] and incubated them at 4°C for 12 h. We removed cell debris by centrifugation and used the



**Fig. 4.** Spatial distribution of *Mc1r* alleles in the wild for *S. undulatus* (derived *Mc1r* allele dominant) and *A. inornata* (derived *Mc1r* allele recessive). Proportion of wild-type (black) and derived (white) alleles across dark soil, ecotone, and white sand habitat. *n*, number of alleles sampled.

supernatant for ELISA. Microtiter plates were coated (at 4°C for 16 h) with a monoclonal antibody directed against the carboxy-terminal Flag tag (Sigma). After blocking (with 10% FBS in PBS), we incubated cell lysates at 37°C for 2 h. We washed plates three times with PBS containing 0.05% Triton X-100 (PBS-T). Thereafter, a peroxidase-coupled anti-HA antibody (3F10; Roche) was added, and plates were incubated at 21°C for 2 h. Then we washed the plates with PBS-T three times with color reaction and took measurements as described above.

#### Understanding the Phenotypic Effects of *Mc1r* Mutations in Nature. Sampling.

To understand the relationship between *Mc1r* genotype, color phenotype, and their spatial distribution in nature, we collected additional genotypic and phenotypic data for the two species with evidence for functional disruption of the blancher *Mc1r* allele (*S. undulatus* and *A. inornata*). We sampled three habitat categories: (i) "white sand" localities are in the heart of the White Sands formation and have white gypsum substrate; (ii) "dark soil" localities are allopatric to white sands and have brown adobe substrate typical of the Chihuahuan Desert region; and (iii) "ecotone" localities are parapatric to white sands and are areas of transition from dark soil to white sands substrate. For each species, 12–21 adult individuals per habitat type were sampled:  $n = 39$  for *A. inornata* and  $n = 52$  for *S. undulatus* (sample numbers and collecting localities are listed in Table S3).

**Color quantification.** For each sample, we took spectrophotometric readings to measure dorsal color variation (following ref. 5). Briefly, dorsal coloration was characterized by averaging spectrophotometric readings from three points along the dorsal midline using an Ocean Optics USB 2000 spectrophotometer with a dual deuterium/tungsten halogen light source. A custom-made probe holder was used to orient the probe at 45° and 1 cm away from the dorsal body surface. We took each spectral reading in reference to a white standard and calculated percent transmission at 0.3 nm intervals. We used readings from 400 to 700 nm, the visual spectrum, for analysis. Previous analyses have shown that color differences among lizards

in this system are explained primarily by differences in brightness (intensity of light transmission), as opposed to hue or chroma (5). Therefore, we focus on a direct estimate of brightness from the spectrophotometric data: area under the spectral curve (AUC).

***Mc1r* genotyping.** To obtain *Mc1r* genotypes, we extracted whole genomic DNA from frozen tissue with Qiagen DNeasy extraction kits. The entire coding region of *Mc1r* was amplified using species-specific primers and conditions reported in ref. 6. Using an ABI3730 (Applied Biosystems), we sequenced diploid PCR products in both directions with species-specific primers as well as internal primers universal to reptiles (6). We edited and aligned sequences using Sequencher (Gene Codes). Heterozygous sites were identified by visual inspection of chromatograms and confirmed by sequence from both DNA strands.

**Statistical analysis.** We evaluated the distribution of *Mc1r* alleles in each habitat and the relationship of color phenotypes to *Mc1r* genotype. For each species, we conducted an ANOVA to compare coloration (i.e., AUC brightness scores) for individuals in each genotypic class (homozygous wild-type, homozygous derived, heterozygous). The goal of this analysis was to determine (i) whether individuals with different *Mc1r* genotypes exhibit different color phenotypes and (ii) the likely dominance relationship between *Mc1r* alleles. If an ANOVA was significant, we used post-hoc Tukey HSD tests to determine which genotypic classes differed significantly in brightness. Statistical analyses were executed in Statistica (StatSoft).

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