Coat Color Variation in Rock Pocket Mice (*Chaetodipus intermedius*): From Genotype to Phenotype

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In a series of classic studies in mammalian evolutionary biology, Sumner (1921), Benson (1933), and Dice and Blossom (1937) described striking coat color variation in the rock pocket mouse, *Chaetodipus intermedius*, in the deserts of Arizona and New Mexico. These authors showed that *C. intermedius* coat color typically matches the color of the rocks on which the mice live; the dorsal pelage varies from a light, sandy color for populations found on some granites to a dark, nearly black color for populations found on basalt lava flows. Dice and Blossom (1937) suggested that this crypsis is an adaptation to avoid predation. Motivated by the wealth of data on the genetics, biochemistry, and molecular biology of the pigmentation process, we have used a candidate-gene approach to identify the genetic basis of adaptive coat color variation in *C. intermedius*. We review our recent studies on this topic with emphasis on the following key results: the identification of a single gene (the melanocortin-1-receptor, *Mc1r*) in one population that appears to be largely responsible for color differences, the balance between selection and migration among neighboring melanic and light races, and the finding that melanism has evolved independently on different lava flows through changes at different genes.

ADAPTIVE COAT COLOR VARIATION IN RODENTS

Coat color variation in small mammals is a classic example of phenotypic variation in response to selection in different environments; many species closely match the color of the substrate on which they live. This geographic variation in phenotype is well documented both within and between species. Examples of intraspecific color variation in rodents include the canyon mouse (*Peromyscus crinitus*), the deer mouse (*Peromyscus maniculatus*), the oldfield mouse (*Peromyscus polionotus*), Botta's pocket gopher (*Thomomys bottae)*, and the rock pocket mouse (*Chaetodipus intermedius*). Although there has been some controversy concerning the importance of crypsis in maintaining this kind of variation (Sumner, 1921; Dice and Blossom, 1937), studies in *Peromyscus* have clearly demonstrated that owls discriminate between mice that do and do not match the color of their substrate (Dice 1947; Kaufman 1974).

At the molecular level, pigmentation is one of the best-studied phenotypic traits in mammals (Silvers, 1979). We have a reasonable understanding of the developmental pathways involved in the formation of melanocytes, the specialized

cells that are the site of melanin production, and of the biochemistry of melanin synthesis (Urabe et al., 1993). Importantly for our work, many of the genes underlying these processes have been identified, primarily from studies in the laboratory mouse. Many coat color mutants are known, and over twenty have now been characterized at the molecular level and associated with specific nucleotide changes. With this information, we have been able to target candidate genes that generate phenotypes similar to those seen in wild populations of rodents in an attempt to identify genes involved in adaptive coat color differences.

Here, we review our recent studies on the genetic basis of coat color variation in the rock pocket mouse, *Chaetodipus intermedius* (Rodentia: Heteromyidae). This species of desert-dwelling rodent displays one of the most striking examples of pigmentation variation in mammals, with light and melanic animals inhabiting granitic and volcanic substrates, respectively. This system offers a unique opportunity to investigate the genetic basis of an adaptive phenotype. First, we describe the natural history of rock pocket mice and observed patterns of phenotypic variation. Next, we review the genetics and biochemistry of mammalian pigmentation pathways. We then focus on several key results from our recent work, namely (1) the discovery of a gene that underlies major color differences in one population, (2) our estimates of the strength of selection on this gene, and (3) our finding that similar melanic phenotypes have evolved independently on different lava flows through changes at different genes.

Phenotypic Variation in *Chaetodipus intermedius*

C. intermedius inhabits rocky areas and desert scrub at low elevations in the southwestern deserts of North America, ranging from southern Utah through most of western and southern Arizona, southern New Mexico, western Texas and adjacent areas in northern Mexico. Rock pocket mice are primarily nocturnal, and show diminished activity from November through February. While pregnant mice have been caught at all times of the year, the peak of reproductive activity is June (Reichman and Van de Graaff, 1973). Females can have as many as three litters per year (Brown and Harney, 1993). Young are born naked, usually attaining their first coat at two to three weeks; the first coat is generally thinner and grayer than the adult pelage (Eisenberg, 1993). Young begin to forage outside the burrow at about one month of age and the molt to adult pelage is thought to begin at 8-10 weeks (Eisenberg, 1963). While there are no studies of predation specifically on *C. intermedius*, it is well established that desert heteromyids, in general, are preyed upon by owls as well as snakes and mammalian carnivores (Brown and Harney, 1993).

Throughout most of their range, adult pocket mice have sandy dorsal pelage and nearly white underbellies. Individual hairs on the dorsum are banded, and the ventral hairs are uniformly light colored, as is typical for many rodent species.

However, several populations of rock pocket mice have been described that have strikingly different pigmentation patterns (Figure 1). Dice (1929) described nearly black (melanic) mice on the Carrizozo lava beds of the Tularosa Basin of central New Mexico (Figure 2). Geographically isolated populations of melanic mice have also been reported on other lava beds (Sumner, 1921; Benson, 1933; Dice and Blossom, 1937). In a comprehensive study, Dice and Blossom (1937) surveyed *C. intermedius* coat color variation throughout Arizona and New Mexico. By quantifying light reflectance of dorsal hairs and the surrounding substrate, they demonstrated a strong correlation between the color of the pelage and the color of the substrate on which the mice live. Experiments conducted by Dice (1947) showed that substrate matching in *Peromyscus maniculatus* protected mice against predation from long-eared owls and barn owls. Although comparable experiments have not been conducted with *C. intermedius*, it seems reasonable to expect similar results, since the degree of color variation is greater in *C. intermedius* than in *P. maniculatus*.

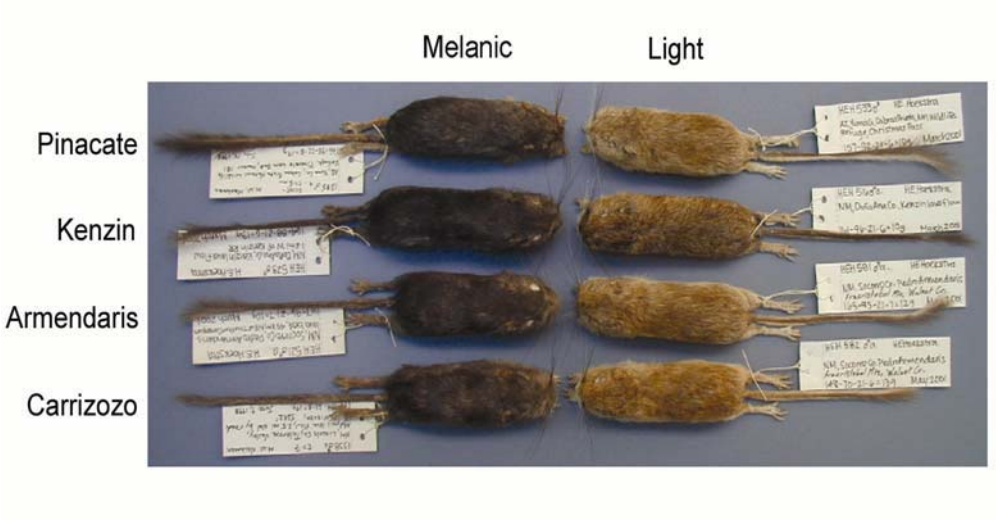

Figure 1. Coat color variation within rock pocket mice, *Chaetodipus intermedius*. The melanic mice in each pair were caught on lava, while the light mice in each pair were caught on nearby light-colored rocks.

While it has been suggested that the color morphs of *C. intermedius* represent different "races" or even subspecies of *Chaetodipus* (Williams et al., 1993), Hoffmeister (1986) noted that phenotypic variation in color is not correlated with morphological variation. In our studies, we found no correlation between coat color variation and mitochondrial DNA variation; instead, we found a strong concordance between mitochondrial phylogeny and geography, independent of coat color (Hoekstra, Krenz and Nachman, 2005). In other words, geographically proximate populations tend to be closely related and share morphological similarities, but can vary dramatically in coat color.

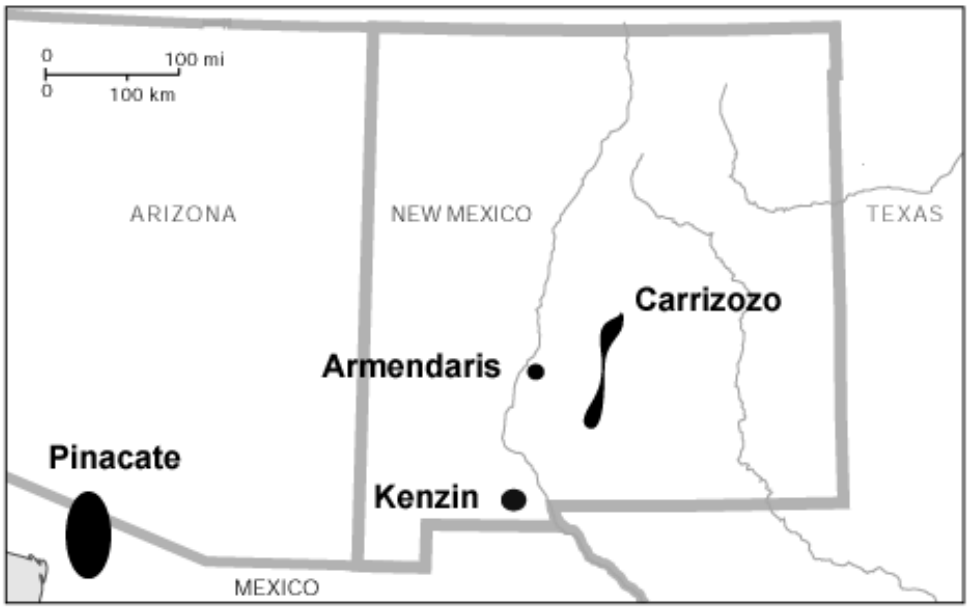

Figure 2. Map of the four lava flows sampled in our studies. Other populations of melanic *C. intermedius* have been identified but are not shown on this map.

The Mammalian Pigmentation Process and Candidate Genes

Pigmentation genes have served as a model system for exploring gene action in a variety of biological processes and for understanding the genetics of mammalian biochemistry, cell biology, and development (reviewed in Silvers, 1979; Jackson, 1994; Barsh, 1996; Jackson, 1997). The deposition of pigment in hair and skin is the culmination of a complex process that involves the coordinated action of many

genes and cell types. Melanocytes, the pigment-producing cells, originate in the neural crest and migrate during development throughout the dermis. The melanoblast cell lineage that gives rise to melanocytes is committed early in development, and subsequent expression of many gene products is regulated in a cell-specific manner (Steel et al., 1992; Erickson, 1993; Bronner-Fraser, 1995). Within melanocytes are specialized organelles known as melanosomes (reviewed in Prota, 1992), which are the site of melanogenesis. There are two primary types of melanosomes, and they differ both structurally and biochemically. Eumelanosomes are ellipsoidal and are the site of synthesis of black or brown eumelanin; phaeomelanosomes are spherical and are the site of synthesis of yellow or red phaeomelanin. Once full of melanin, melanosomes are secreted from the melanocyte as pigment granules. Several lines of evidence suggest a close relationship between melanosomes and lysosomes, and it is possible that melanosomes are modified lysosomes (Jackson, 1994, 1997). For example, many mouse mutations, which affect melanosome function, also disrupt lysosome function (e.g. Barbosa et al., 1996; Feng et al., 1997). From an evolutionary perspective, this highlights one of the many ways in which pigmentation mutants may have important pleiotropic consequences. Finally, synthesis of melanin within melanosomes involves the interactions of many loci, and some aspects of melanogenesis are under hormonal regulation.

Pigmentation mutations in the laboratory mouse have been identified in all steps of this complex process (Prota, 1992; Jackson, 1994). For example, there are mutant phenotypes such as *piebald*, *steel*, and *white spotting* that result from improper development or migration of melanocytes, leaving portions of the body without pigment-producing cells. Other mutations, such as *beige* and *pale ear*, interfere with the proper structure and function of melanosomes. Some mutations, such as *albino*, *brown*, or *slaty*, interfere directly with proteins involved in synthesis of melanin. Finally, mutations at the *agouti*, *extension*, and *mahogany* loci disrupt the control and regulation of melanogenesis. Approximately 80 genes have been identified that affect coat color in the laboratory mouse and approximately one-quarter of these have been cloned, sequenced, and characterized at the molecular level (Jackson, 1997). The availability of genes known to affect each of the steps leading to the deposition of melanin in the laboratory mouse presents an opportunity to investigate the genetic changes underlying phenotypic differences in pigmentation in natural populations of rodents.

Several loci involved in pigmentation are particularly likely candidates for the coat color differences seen among populations of *C. intermedius*. A key distinction in melanogenesis is the production of eumelanin (black or brown pigment) versus phaeomelanin (yellow or red pigment). This difference is controlled in large part by the interaction of three proteins: the Agouti signaling protein, alpha-melanocyte stimulating hormone (α-MSH) and melanocortin-1 receptor (MC1R) (Figure 3). MC1R is a transmembrane G-protein-coupled receptor that is highly expressed in

melanocytes. Melanocyte-stimulating-hormone activates MC1R and results in elevated levels of cAMP and increased production of eumelanin. Agouti is an antagonist of MC1R; local expression of Agouti results in suppression of synthesis of eumelanin and increased production of phaeomelanin. In the laboratory mouse, multiple alleles have been identified at both *Agouti* and at *Mc1r*, and mutations at both loci produce a range of phenotypes from dark to light color. Dominant *Agouti* mutations result in increased Agouti expression and largely yellow phenotypes. In contrast, recessive, loss-of-function *Agouti* mutations result in nonagouti, all black

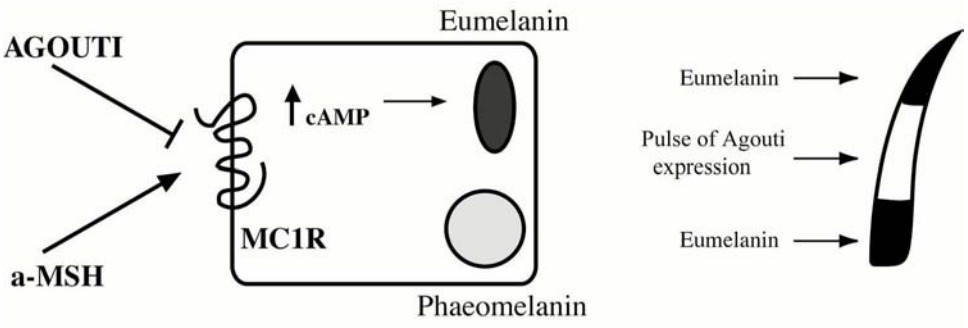

Figure 3. Regulatory control of mammalian melanogenesis. Alpha melanocyte stimulating hormone (α-MSH) signals MC1R, resulting in the up-regulation of cAMP and synthesis of eumelanin. The Agouti protein is an antagonist of MC1R that results in the default pathway of phaeomelanin production. Individual hairs on the dorsal surface of light-colored *C. intermedius* are banded, as shown on the right. In the laboratory mouse, this banded pattern is known to result from a pulse of Agouti expression in individual hair follicles during the middle of the hair cycle. Melanic *C. intermedius* have unbanded dorsal hairs (not shown).

phenotypes. Dominance relationships among *Mc1r* alleles are opposite in effect to those at *Agouti*, meaning that recessive, loss-of-function *Mc1r* mutations typically result in yellow phenotypes (although slightly different phenotypically from the dominant yellow of *Agouti*). Agouti expression varies both spatially and temporally (Bultman et al., 1992; Siracusa, 1994). Wild mice have light bellies as a result of constitutive ventral Agouti expression and associated production of phaeomelanin. In contrast, hairs on the dorsum of wild mice have a banded pattern, with a black tip, a middle yellow band, and a black base (the agouti hair, Figure 3). This banding derives from a pulse of Agouti expression during the mid-phase of the hair cycle, resulting in deposition of phaeomelanin during the middle of hair growth and deposition of eumelanin at the beginning and end of hair growth. The

temporal and spatial patterns of Agouti expression are under the control of different promoters (Siracusa, 1994; Vrieling et al., 1994). A mouse mutation known as black-and-tan results from a large insertion in the first intron of the *Agouti* gene (Bultman et al., 1992; Bultman et al., 1994). This insertion eliminates dorsal expression of Agouti but has no effect on ventral expression. The phenotype of these mice includes a light belly and all-black, unbanded dorsal hairs. Similarly, some mutations at *Mc1r* in the laboratory mouse result in dark, unbanded hairs on the dorsum but light hairs on the ventral surface (Robbins et al., 1993).

C. intermedius display a variety of color variants (Figure 1). Many of the melanic animals show similarities to *Agouti* or *Mc1r* mutants in the laboratory mouse in having dark, unbanded dorsal hairs and light bellies. These similarities suggested to us that *Agouti* or *Mc1r* might be responsible for the observed color variation in natural populations. Moreover, at several localities, both light and dark *C. intermedius* are found together without phenotypic intermediates, suggesting that pigmentation differences may be controlled by a few genes of major effect, although it is important to bear in mind that strong selection against intermediates or positive assortative mating could also produce this pattern.

We have used association studies and a candidate-gene approach to identify the genes underlying color variation in *C. intermedius*. The general strategy is to develop single-nucleotide-polymorphism (SNP) markers for each candidate gene. These SNPs are then surveyed in geographically adjacent populations of light and dark mice. If a strong association between SNP variants and coat color phenotype is found, we sequence the entire gene in all light and dark individuals and test for population structure by looking at additional, unlinked markers. One advantage of association studies over laboratory crosses is that natural populations will have undergone many more generations of recombination; thus if an association is detected, it is likely that the markers are relatively close to the functional sites.

THE ROLE OF *MC1R* ON COLOR PHENOTYPE IN THE PINACATE LAVA FLOW

We sampled mice from paired localities in four different regions (Tables 1 and 2, Figure 2). Each region contains a lava flow, and in each region we have sampled mice from the dark lava and from adjacent areas with light-colored rocks. Consistent with earlier work by Dice and Blossom (1937) from many of these same localities, we found a strong association between substrate color and coat color. Here, we concentrate on one well-sampled region, the Pinacate lava bed, which is situated in northern Sonora, Mexico, and the adjacent Cabeza Prieta National Wildlife Refuge in southern Arizona (Figure 2). This lava flow is estimated to be approximately 1.7 million years old (Lynch, 1989), and many of the rocky areas are disjunct due to the accumulation of intervening deposits of sand.

In our first study (Nachman et al., 2003), 29 mice were collected from two sites - one on the dark lava and one on the nearest light rocks (the rocky slopes of the O'Neill Hills, approximately four kilometers east of the lava). Of the 18 mice captured on dark rock, 16 were dark or melanic (89%); while 10 of 11 mice (91%) caught on the light rock were light. We found no association between coat color phenotypes and *Agouti* SNPs. However, several *Mc1r* SNPs revealed a perfect association with coat color phenotypes. The association observed at several *Mc1r* SNPs led us to characterize the entire *Mc1r* gene in *C. intermedius*. PCR primers were designed from conserved regions in the alignment of human and *Mus Mc1r* sequences, and then genome-walking was used to capture the 5′ and 3′ ends of the gene. *Mc1r* has a single coding exon of 954bp (318 amino acids); this simple gene structure is conserved between birds and mammals. We cloned and sequenced the 58 alleles from our sample of 18 melanic and 11 light mice.

Table 1. Sample sizes of *C. intermedius* at each of the four lava flows in Figure 2.

	Mouse coat color	
	Light	Melanic
Pinacates	123	102
Armendaris	12	8
Carrizozo	4	8
Kenzin	6	14

Table 2. Estimated age of oldest lava flow, largest contiguous area of lava, and distance to nearest light rock (Lynch, 1989; Hoffer and Corbitt, 1991).

	Age (yrs)	Area (km^2)	Distance (km)
Pinacates	1,700,000	3500	4
Armendaris	760,000	435	5
Carrizozo	1,000	329	20
Kenzin	530,000	142	7

Several lines of evidence suggest that mutations at *Mc1r* are responsible for the observed phenotypic differences in color in mice from the Pinacate Lava Beds (Nachman et al., 2003). First, a perfect association is seen between four amino acid polymorphisms and coat color. These four amino acid polymorphisms are in complete linkage disequilibrium with one another and thus create a single haplotype (which we refer to here as the melanic allele). Mice with one or two copies of the melanic allele are melanic, while mice without this allele are light (Table 3). This perfect association is highly significant (Fisher's Exact test, $p < 0.0001$). Such an association is expected if *Mc1r* is responsible for the observed coat color differences. However, such an association might also arise if a gene tightly linked to *Mc1r* is responsible for the phenotype, rather than *Mc1r* itself. Genomic sequences and genetic maps from *Mus* and humans suggest that there are few genes neighboring *Mc1r*. Moreover, there are no known genes involved in pigmentation adjacent to *Mc1r* in either species. To further test the hypothesis that *Mc1r*, rather than a linked region, is contributing to the observed phenotypic variation, we are currently using genome-walking to determine the extent of linkage disequilibrium surrounding *Mc1r*. This will delimit the genomic region associated with the phenotype. Preliminary results indicate that linkage disequilibrium decays within 2 kb both upstream and downstream of *Mc1r*, thus ruling out the involvement of a linked locus (Hoekstra et al, in prep). An additional concern with association studies comes from the potential for population structure to create spurious associations between genotypes and unrelated phenotypes (Lander and Schork, 1994; Lynch and Walsh, 1998). The complete ND3 and COIII mitochondrial DNA genes were sequenced in all 29 animals to test for population structure. Patterns of variation at these loci were consistent with a single interbreeding population of light and melanic mice, suggesting that population structure is not responsible for the strong association between *Mc1r* alleles and coat color phenotypes.

A second observation that suggests a direct role for *Mc1r* is that all four amino acid mutations on the melanic allele result in a change of charge and occur at nucleotide sites that may be important for receptor function. These four mutations are derived relative to *Mc1r* sequences in the sister species, *C. penicillatus*, which is light in color. Two mutations are found in extracellular regions and may be important for ligand binding, while two are found in intracellular loops and may be important for G-protein interactions (Figure 4). Previous studies in other species have identified single amino acid changes at *Mc1r* that result in darkening mutations; these mutations have been identified primarily in intracellular regions or at the boundary of transmembrane and extracellular loops (Figure 4).

Third, the dominance pattern seen in *C. intermedius* follows the pattern predicted from studies of the laboratory mouse: dark *Mc1r* alleles are dominant over light alleles. Individual *C. intermedius* that are heterozygous or homozygous for the melanic allele have a dark phenotype. In laboratory mice, gain-of-function

mutations at *Mc1r* are dominant, whereas loss-of-function mutations at *Mc1r* are recessive (Robbins et al., 1993).

Finally, overall patterns of nucleotide variability at *Mc1r* show evidence of recent, strong directional selection (Nachman et al., 2003). Comparison of the level of nucleotide variability among the melanic alleles to the level of variability among the light alleles shows a significant reduction in polymorphism among melanic alleles; there is only a single polymorphic nucleotide segregating among the melanic alleles while there are thirteen variable sites among the light alleles. The average level of nucleotide heterozygosity, π, is 0.01% for the melanic alleles and 0.21% for the light alleles, representing a twenty-fold difference. This pattern is consistent with positive, directional selection having acted recently to raise the frequency of the melanic allele. The absence of genetic variation is consistent with hitchhiking effects on linked, neutral sites. Further evidence of genetic hitchhiking comes from the observation that a single silent site is in complete linkage disequilibrium with the four amino acid mutations on the melanic allele.

Together, these observations strongly implicate *Mc1r* in the melanic phenotype in the Pinacate population, and provide a rare example of the molecular changes underlying an adaptive phenotype in a natural population. It remains unclear whether one or more of the four amino acid variants on the melanic allele contribute to the observed phenotypic differences; this is currently being addressed through *in-vitro* functional studies as described below.

Table 3. Association between *Mc1r* genotype and coat color in *C. intermedius* from the Pinacate lava flow. Melanic allele (*D*) and light allele (*d*).

	Phenotype	
Mc1r genotype	Light	Melanic
DD	0	11
Dd	0	6
dd	12	0

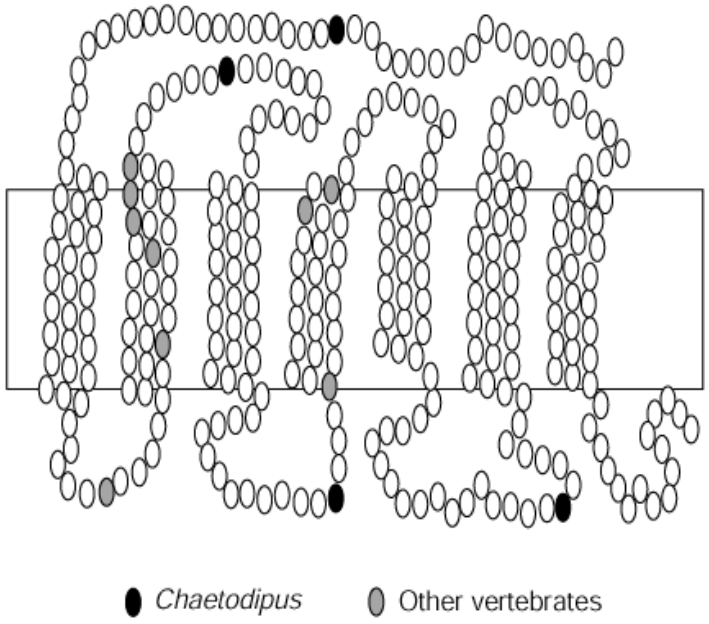

Figure 4. Structure of the MC1R protein. The receptor consists of an extracellular N-terminal domain, seven transmembrane regions separated by intracellular and extracellular loops, and a C-terminal intracellular domain. Gray circles identify amino acid positions at which darkening mutations have been identified in other vertebrate species (mouse, fox, dog, cow, sheep, chicken). Black circles identify the four amino acid positions that differ between light and melanic alleles in *C. intermedius*.

The Balance Between Migration and Selection

Identification of a gene underlying phenotypic variation in pocket mice from the Pinacate Lava Flow allowed us to investigate spatial variation in genotype frequencies and to estimate the strength of selection from a simple model of migration-selection balance. We investigated the pattern of genotypic and phenotypic change across geography by conducting a 30 kilometer transect across both light and dark rocks in the Pinacate region (Figure 5) (Hoekstra et al., 2004). This transect included three sites on dark rock and three sites on light rock. The average distance between sites was approximately six kilometers. Except on the

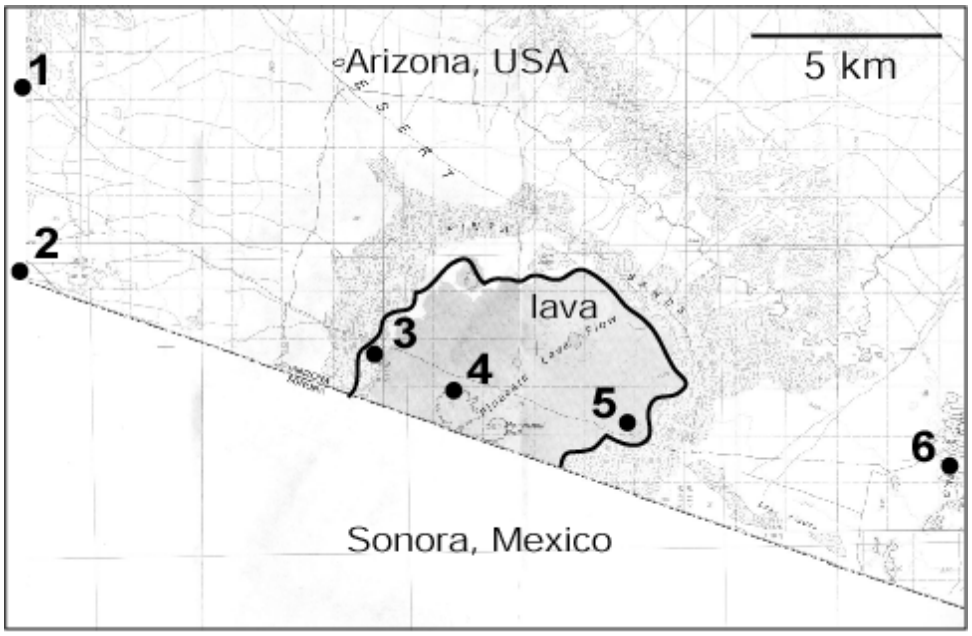

Figure 5. Transect across the Pinacate lava flow. Six sampling sites are indicated: three on volcanic basalt, and three on light-colored granite. Each site is separated by approximately six kilometers.

lava, much of the habitat between sites was dominated by light-colored sand, which is not suitable for *C. intermedius*. In total, 225 mice were sampled. Fifty-seven mice were captured on dark rock; 94.7% of these had a melanic phenotype. One hundred sixty-eight mice were captured on light rock; 71.4% of these had a light phenotype. Across all sites, we found a strong correlation between habitat color and coat color of mice (Figure 6). Interestingly, the correlation between substrate and coat color was stronger on the western side of the lava flow than on the eastern side. The increased frequency of "mis-matched" mice on the eastern edge of the lava may be due to the closer proximity between light and dark habitats, facilitating migration between them.

To determine the distribution of *Mc1r* alleles across this transect, we sequenced the entire *Mc1r* coding region (954bp) for 202 individuals (Hoekstra et al., 2004). Consistent with our earlier studies based on smaller samples, we found that (1) all melanic individuals had at least one melanic allele with the four amino acid variants, (2) there was a strong correlation between *Mc1r* allele frequency and habitat color, and (3) there was no correlation between habitat color and neutral

mtDNA markers. This larger sample further strengthened the association between *Mc1r* genotype and coat color phenotype. The strong correlation between *Mc1r* alleles and substrate color, in the face of gene flow observed in patterns of mtDNA variation, suggests that differential selection on different substrates is acting to maintain the color polymorphism.

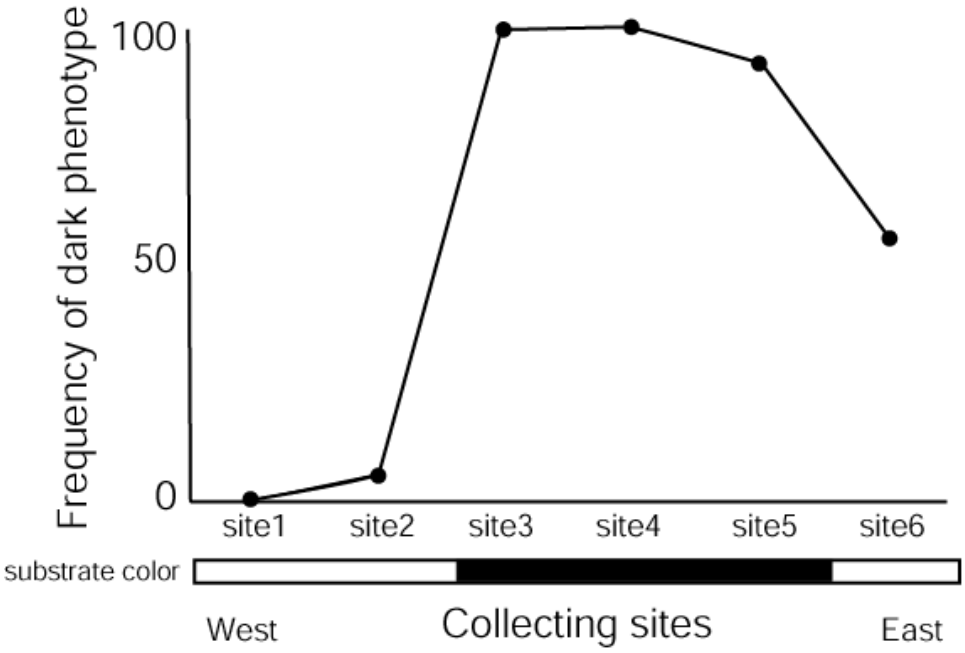

Figure 6. Correlation between coat color of *C. intermedius* and substrate color. Frequency of melanic individuals caught at each of the six sampling sites in Figure 5. Substrate color is shown schematically below.

A simple model of migration-selection balance can be used to estimate the strength of selection in this situation. We assume that the frequency of melanic alleles on light substrate (and the frequency of light alleles on dark substrate) is at steady-state, maintained by the input of new mutations through migration and their elimination by selection. This model further assumes that effective population sizes are equivalent among sites, that mutation is negligible, and that migration occurs between the sites surveyed (as opposed to migration from other sites). Under these assumptions, the equilibrium frequency of a dominant mutation, p, is given by m/s, and the equilibrium frequency of a recessive mutant, q, is given by $\sqrt{m/s}$, where m is the per-generation migration rate and s is the average selection coefficient. By

estimating p, q, and m we can then derive a very rough estimate of s (Figure 7). This simplified model ignores emigration and assumes that all *Mc1r* alleles introduced by immigration are deleterious. For a more complete treatment of this topic, see Hoekstra et al. (2004).

Here, we estimate s by considering gene flow between the Pinacate lava and a neighboring light locality, the O'Neill Hills (sites 3 - 5 and 6 in Figure 5). Samples from the three sites on the Lava (sites 3 – 5) were combined because of their close proximity and the lack of population structure between these sites. The total sample contains 77 mice from the light site and 57 mice from the dark site. Thirty-four of 43 mice (79%) from the granite were light, while 55 of 57 mice (96%) from the lava were dark. Gene flow between these sites was estimated by comparing mitochondrial sequence variation for the ND3 and COIII genes using the program MIGRATE (Beerli, 1999; Beerli and Felsenstein, 2001). MIGRATE calculates a maximum likelihood estimate of N_em between populations using a coalescent approach, relaxing the traditional assumption of symmetrical migration. By

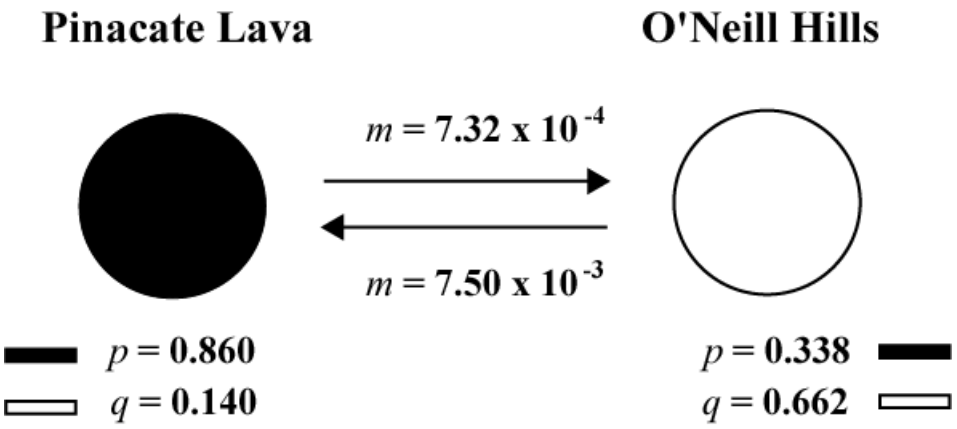

Figure 7. Migration-selection balance between populations of *C. intermedius* on dark and light substrates (corresponding to sites 5 and 6 in Figure 5, respectively). Migration rates (m) were estimated from mtDNA. Frequencies of *Mc1r* melanic alleles (p) and *Mc1r* light alleles (q) at each site are shown.

quantifying asymmetric migration rates, we can estimate selection against light mice on dark rocks as well as selection against dark mice on light rocks (Figure 7). The maximum-likelihood estimate of N_em from the Lava to O'Neill Hills was 7.32, while the maximum-likelihood estimate of N_em from O'Neill Hills to the Lava was 75.03. We estimated N_e from the level of mitochondrial nucleotide heterozygosity (π

= 0.01), under the neutral expectation $\pi = N_e\mu$, assuming $\mu = 10^{-6}$ and assuming a sex ratio of one. This suggests that, approximately, $N_e = 10^4$, which is consistent with estimates from other small rodents (e.g. Nachman 1997). This provides an estimate of $m = 7.32 \times 10^{-4}$ from the Lava to O'Neill Hills, and $m = 7.50 \times 10^{-3}$ from O'Neill Hills to the Lava, implying that migration from light rock to lava is higher than migration in the opposite direction. The frequency of the *Mc1r* melanic allele was $p = 0.338$ at O'Neill Hills and the frequency of the light allele was $q = 0.140$ on the Lava. These calculations provide an estimate of $s = 0.002$ for melanic mice on light rocks (O'Neill Hills) and $s = 0.383$ for light mice on dark rocks (Lava). Although these numbers are crude and are based many untested assumptions, they suggest that selection may be stronger against light mice on dark rocks than against melanic mice on light rocks.

Interestingly, the larger estimate of s is associated with the evolution of the novel phenotype; the melanic allele and the dark color are derived relative to the sister species, *C. penicillatus*. Thus, light mice initially encountered and colonized dark rock, and strong selection led to the proliferation of the melanic phenotype. These asymmetrical selection coefficients also parallel observations from peppered moths, in which there was a rapid increase in melanic moths, but a comparatively slow decrease in melanic moths when the habitat became lighter in color as levels of pollution decreased (Kettlewell, 1955). These observations suggest that selection was stronger against light moths on dark background than against dark moths on light background. This pattern may be driven by a bias in avian visual systems in which light objects on dark backgrounds are more conspicuous than the reverse.

Role of *Mc1r* in Other Melanic Populations

A fundamental question in evolution centers on the issue of genetic constraints to adaptive change: given a common evolutionary problem, are there multiple genetic solutions? Having discovered that *Mc1r* appears to play a major role in the color phenotype in the Pinacate population, we were interested in exploring whether similar phenotypes in other populations have evolved independently and, if so, whether they have evolved via changes at the same gene or via changes at different genes (Hoekstra and Nachman, 2003). To explore this issue, we sampled mice from pairs of light and dark localities in four different regions (Table 1, Figure 2). The four different lava flows vary in both size and age (Table 2) and, thus, mice on the lava flows may have very different population histories.

Several observations suggest that similar melanic phenotypes may have evolved independently on different lava flows. First, Hoffmeister (1986) argued there was little gene flow between different melanic races, in part because of the large distances separating many of these populations. Consistent with this, our survey of mitochondrial DNA variation from these four regions revealed a strong correlation between phylogeny and geography, indicating that there has been

relatively little gene flow over this geographic scale (Hoekstra et al., 2005). Additionally, subtle differences in phenotype from different lava beds (Dice and Blossom, 1937) suggest that the melanic phenotype may have a different genetic basis. We found that mice from the Pinacates were slightly grayer than populations from Carrizozo and Armendaris, although these differences were quite subtle. There may also be phenotypic differences within lava flows. We observed two types of melanic mice at the Kenzin lava flow, one with completely melanic dorsal hairs and a second type with banded dorsal hairs in which the light band of phaeomelanin was greatly reduced relative to typical light mice. Both limited gene flow among lava flows and phenotypic variation within and among lava flows suggest that there may be multiple genes involved in generating melanic phenotypes, and that melanic color may have evolved independently several times.

To address this question more directly, we sequenced the *Mc1r* coding region in five light and five melanic mice from each of the localities in Figure 2. None of the four *Mc1r* amino acid variants from the Pinacate population were found in melanic mice from any of the other populations. Moreover, no new *Mc1r* mutations showed an association with phenotype in any of the localities (Hoekstra and Nachman, 2003). There is thus no evidence that *Mc1r* is involved in coat color variation in the Pedro Armendaris, Carrizozo, or Kenzin lava flows. These results indicate that a similar melanic phenotype has evolved independently on different lava flows, and has done so via different genetic changes.

FUTURE DIRECTIONS

Our work to date has demonstrated that mutations at *Mc1r* are responsible for adaptive coat color differences in one population of *C. intermedius* and that similar melanic phenotypes have evolved independently in different populations through changes at different genes. This work, which links genotype with phenotype for a trait of clear ecological importance, has raised a number of questions, and our current efforts focus on two of these issues.

First, we are interested in identifying the specific mutation or mutations at *Mc1r* that are responsible for the phenotypic differences observed in the Pinacate population. Because there are four amino acid changes that distinguish all melanic alleles from all light alleles, we do not know whether one or more of these mutations are responsible for the functional differences. It is possible that each mutation contributes a small amount to the observed phenotype, or that two or more mutations interact epistatically to produce the phenotype. Alternatively, some of the mutations may be neutral, having hitchhiked along with the selected mutation(s). To identify the sites of functional importance, we are using an *in-vitro* expression system to measure the function of receptors that have combinations of one or more mutations. Functional assays have been used successfully in *Mus* to show that a single amino acid variant constitutively activates the *Mc1*-receptor in

the case of the somber-3J mutation (Robbins et al., 1993). Functional in-vitro studies using *C. intermedius Mc1r* sequences should allow us to identify the specific mutations responsible for adaptive coat color differences in one population of this species.

Second, we are interested in identifying the genes involved in coat color variation in populations where *Mc1r* is clearly not playing a role. Towards this end, we are currently screening SNPs in candidate genes for each of the populations in Figure 2. The presence of several geographically isolated lava flows, and the independent evolution of melanic phenotypes in these areas, provides an excellent opportunity for replicate evolutionary studies. We hope that from these replicates, some generalities about the genetics of adaptation may emerge, including such things as patterns of dominance, epistasis and pleiotropy, the number of mutations contributing to the observed phenotypes, and whether mutations are in coding regions, regulatory regions, or both.

CONCLUSIONS

With the complete DNA sequence of many species now available, it is becoming increasingly possible to make the link between genotype and phenotype for fitness-related traits. In principle, many of the approaches used here for studying pigmentation differences in *C. intermedius* could be extended to other phenotypes of ecological importance in other eutherian mammals. Our work provides evidence for the genetic basis of a key adaptation in a natural population of rodents. It appears that similar melanic phenotypes have arisen independently in *C. intermedius* populations on different lava flows and that these changes have occurred through changes at different genes. In this situation there appears to be more than one genetic solution to a common evolutionary problem.

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