

ENVIRONMENTAL AND GENETIC INFLUENCES ON MELANIN-BASED PLUMAGE
COLORATION: IMPLICATIONS FOR POPULATION DIVERGENCE

by

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A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Ecology and Evolutionary Biology

2014

This thesis entitled:
Environmental and genetic influences on melanin-based plumage coloration: implications for
population divergence
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Environmental and genetic influences on melanin-based plumage coloration: implications for
population divergence
Thesis directed by Associate Professor Rebecca J. Safran

ABSTRACT

Colorful traits in animals often function to attract or compete for mates. However, the information gained by receivers (potential mates and competitors) is often unknown. Mechanisms of sexual selection (mate choice via indirect vs. direct benefits) make different predictions about the type of information provided by these traits and therefore whether trait expression is primarily influenced by genetic (indirect benefits) or environmental (direct benefits) variation. The goal of my dissertation research was to assess the role of genetic and environmental variation on melanin-based coloration and how these influences vary in response to different selective pressures. I conducted several field studies using two phenotypically divergent populations of barn swallows, *Hirundo rustica*. First, in the North American barn swallow (*H.r. erythrogaster*) in which ventral coloration is known to influence reproductive success, I used longitudinal data to demonstrate that ventral plumage coloration within an individual was consistent across developmental stages. Next, I conducted a cross-fostering experiment to tease apart the genetic and environmental influences on plumage color development in this subspecies. I found that coloration is quite sensitive to environmental variation, with low heritability, suggesting females use this trait in mate choice to assess direct benefits provided by a mate. Finally, I replicated this cross-fostering experiment in the Czech Republic with a different subspecies of barn swallows (*H.r. rustica*) where the role of coloration in mate choice is unknown. I found that the relative genetic and environmental influences on color were similar in this divergent population; however, the genetic covariance structure of

color traits differed. Together, these results demonstrate that coloration is influenced by developmental environment more than genetic environment. Thus, in North America, where females prefer males with dark plumage, coloration serves as a better signal of developmental conditions than genetic quality. Moreover, divergent selection on plumage coloration may explain the phenotypic differences among these populations, suggesting a role of sexual selection in the diversification of the barn swallow species complex.

ACKNOWLEDGMENTS

Conducting and completing my graduate research has been one of the most challenging and fulfilling endeavors of my life. Throughout my time here at CU, I have received help and support from a number of people and I am forever grateful. First and foremost, I would like to thank my PhD advisor, Dr. Rebecca Safran, who has always been generous with her time, always provided constructive feedback, and always cheered me on. With her enthusiasm and passion for science and discovery, Becca has always fostered a productive working environment with high expectations and all the resources to achieve them. Most importantly, Becca's leadership and investment in her students has led to our lab group being a second family for everyone. I am not only leaving the Safran lab with an amazing group of scientific collaborators whom I will continue to work with throughout my career, but also with dear friends whom I will continue to catch up with throughout my life.

I would also like to thank my committee, Dr. Robert Guralnick, Dr. Andrew Martin, Dr. David Stock, and Dr. Kim Hoke, for their time and patience throughout my graduate career. It was always great to receive positive feedback from my committee, especially when I doubted the impact and importance of my research. I feel extremely lucky that I left my exams and my defense in high spirits after great, although sometimes difficult, conversations about how to move forward with my work. My committee has helped me become a better scientist and I know that I can continue to seek their advice and feedback as I move forward in my career.

The Ecology and Evolutionary Biology department at CU is a unique place; I have never heard of another academic department that is as completely supportive and friendly as ours. Throughout my time here, I have had the privilege to interact, both professionally

and personally, with our amazing faculty, postdoctoral researchers, graduate students, undergraduate students, and staff. While the list is too long to include here, I would like to name a handful of people that have been beyond influential. To members of my lab (past and present), thank you for all your inspiration and support: Rachel Bradley, Cait Dmitriew, Roi Dor, Andrew Flynn, Amanda Hund, Brittany Jenkins, Iris Levin, Liz Scordato, Matt Wilkins, and David Zonana. Matt – we had no idea what we were getting into back in August of 2008...thank you for always being there and I look forward to sharing our adventures as we move forward. Amanda – where do I start? So much of my research would have been impossible without your collaboration and support! To my friends outside the lab, thank you for making me take a step back and think about other aspects of biology and for all the science talk over beers: Chelsea Cook, Tommy Detmer, Katie Driscoll, Helen McCreery, Joe Mihaljevic, Se Jin Song, Amy Trowbridge, Ty Tuff, and Sarah Wagner. I would also like to say a special thank you to the many undergraduate field assistants I worked with over the years. Our field season is long and brutal and I appreciate, more than you know, your dedication and hard work!

Outside of my department, I need to thank my international collaborators, without whom, a major component of my dissertation would have been impossible: Dr. Tomáš Albrecht, Romana Michálková, Adela Petrželková, Martina Soudková, and Dr. Oldrich Tomášek. Tomáš, many thanks for allowing Amanda and I to commandeer your study sites for a summer and helping to arrange all the logistics. The extent of your knowledge about everything is astounding! Martina, thank you for introducing us to the Czech Republic and translating so many menus. Adela and Romana, it was such a joy to live and work with you for the months we

were there. Olda, despite your snoring and crazy driving, I learned and laughed a lot from with you in the field.

As a graduate student at CU, I have been involved in the United Government of Graduate Students (UGGS). I want to thank the organization for all they do to advocate for graduate students on campus and providing me with a deeper understanding of how institutes of higher education function. Moreover, through my involvement in UGGS, I met graduate students from a diverse array of disciplines, many of whom are now some of my dearest friends: Kate Allison, Steven Koenig, John Lurquin, and Laura Michaelson. In addition to being such a huge source of support and motivation, you have helped me make a difference while a student on this campus. The work we have done in the past several years has had large, although often unseen, impacts on the lives of graduate students at CU. Thank you for helping me leave behind a legacy that I am truly proud of. And I should also thank the 28th Street Tavern, the Sink, and the Connection where we held many organizational meetings.

Finally, without the loving support of my family I would have never made it this far. To my parents, Dave and Linda Hubbard, thank you for letting me climb trees, catch crawdads, and bring home toads as a kid. My biological curiosity sparked early and with your support and encouragement, it led to me moving across the country for my first research position, then to masters degree, and finally to my PhD. I know you will continue to support and encourage me in the next steps of my career. To my sister and brother-in-law, Dana and Chris Wuensch, thank you for letting me convince your daughters that science is awesome; it is such a joy to see them as they grow into little naturalists!

My dissertation research was funded by research grants from the University of Colorado EBIO department, the Graduate School Beverly Sears fund, and the Graduate School Dean's

Research fund. Additional outside funding came from the Animal Behavior Society, the American Ornithologists Union, the American Museum of Natural History Frank Chapman award, and the Colorado Field Ornithologists. Undergraduates that assisted with my research were supported by the Undergraduate Research Opportunities Program (UROP) and the Biological Undergraduate Research Skills and Training (BURST) program. I would also like to thank the EBIO department and the Graduate School for semester and summer fellowships, respectively; and the United Government of Graduate Students, the Graduate School, and the American Ornithologists Union for travel funding to attend conferences to present my work.

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Chapter 1

Introduction

Colorful traits are often the most obvious feature of an animal. Beyond being eye-catching, color serves a variety of important functions in animals and consequently, is frequently the target of either natural selection, sexual selection, or both (Nachman et al. 2003; Goldstein et al. 2004; Safran & McGraw 2004; Rosenblum et al. 2004; Amundsen & Pärn 2006; Hill 2006; McGraw 2006a; Senar 2006; Protas & Patel 2008; Steiner et al. 2009; Linnen et al. 2009). To truly understand how selection shapes colorful traits and the role they play in phenotypic divergence among populations, we must determine how much phenotypic variation is explained by genetic variation. While plastic or environmentally dependent traits are important and can affect the survival and fitness of an individual, only traits with some genetic basis will have an evolutionary response to selection. Thus, a principle goal of my dissertation was to understand the genetic and environmental influences on melanin-based plumage coloration.

In chapter 2 of my dissertation, I review what is known about genes that affect plumage coloration in vertebrates. I first outline the extensive functions that colorful tissues (hair, feathers, scales, skin, etc.) can provide an animal. It is apparent that colorful traits have evolved for many different uses ranging from sexual signaling (Safran & McGraw 2004; Amundsen & Pärn 2006; Hill 2006; Senar 2006; Protas & Patel 2008) to crypsis (Nachman et al. 2003; Steiner et al. 2009; Linnen et al. 2009) to thermoregulation (Rosenblum et al. 2004; McGraw 2006a; Protas & Patel 2008). Given this diversity of functions, it may not be surprising that multiple genes and other genetic mechanisms exist to create color variation within and among taxa. There are a variety of pigments and structures that underlie animal coloration. These include melanins, carotenoids, pterins, and other rare pigments, as well as structural coloration. While research has

been focused on many different pigments across many systems, it is clear that the genetic basis of melanin-based plumage coloration is far better understood than other types of coloration. Melanin-based color polymorphisms are taxonomically widespread and have been extensively studied in several systems. Most notably, over 100 pigmentation genes that affect the expression of melanin coloration were identified in lab mice (Silvers 1979). Mutations in several of these genes have been associated with polymorphic variation in numerous vertebrates (Hoekstra & Nachman 2003; Mundy 2005; Nadeau et al. 2007; Uy et al. 2009; Mullen et al. 2009); and interestingly, the same mutations have been shown to associate with the same phenotypic changes across diverse taxa, demonstrating the conserved nature of many of these genes (Hoekstra & Price 2004; Hubbard et al. 2010). I also argue that environment plays an important role in the development of several colorful traits, particularly traits that have a clear dietary link like carotenoid-based colors (Hill 1991; McGraw 2006b). Finally, this chapter outlines the need for more studies to link mechanisms of color development to the function of a colorful trait. Chapter 4 of this dissertation aims to make this link for melanin-based plumage coloration, known to be the target of sexual selection via mate choice.

The remainder of my dissertation research focused on quantifying the genetic and environmental influences on melanin-based plumage color within populations and using that information to better understand phenotypic divergence among populations. To approach these questions I used the barn swallow (*Hirundo rustica*) species complex. Barn swallows are a migratory passerine with a Holarctic distribution composed of six described subspecies (Turner 2006; Dor et al. 2010). My research involved two subspecies, a population in Colorado, USA (*H.r. erythrogaster*) and one in Southern Bohemia, Czech Republic (*H.r. rustica*). In the Colorado population, as well as other North American populations, ventral melanin-based

plumage coloration is highly variable and used in female mate choice decisions (Safran et al. 2005), whereas evidence suggests that tail streamer length, rather than coloration, is used for such decisions in European populations (Saino et al. 1997; Møller et al. 1998). Consequently, my study populations differ in both coloration and the selection acting upon it, making them an excellent study system for exploring how coloration acts as a sexual signal, how the underlying genetic variance changes in response to selection, and the potential role of plumage coloration in phenotypic divergence.

In chapters 3 and 4, I focus on the Colorado population of barn swallows with the goal of gaining a complete understanding of genetic and environmental influences on color variation in this subspecies. As in other passerines, juvenile birds disperse or do not survive migration, with the consequence of very few birds returning to their natal habitat as breeding adults. Consequently, I was unable to determine genetic relationships (i.e., full-siblings, half-siblings, etc.) in adults. Therefore, my first project for this dissertation (chapter 3) was to determine how predictive juvenile coloration is of the adult color used in mate choice decisions. A strong relationship between juvenile color and adult color within an individual would allow me to do manipulative experiments on nestlings and infer their effects on adult coloration. Over several breeding seasons, I accumulated a large enough data set of recruited nestlings to identify a strong relationship in color between developmental stages. Also in chapter 3, I estimated heritability, environmental effects, and maternal effects of color. I took advantage of a naturally high rate of extra-pair young and used an animal model to partition phenotypic variation into these three components. I found that environmental and maternal effects explained a large proportion of phenotypic variation, which does not align with the widely held idea that melanin-based coloration is primarily influenced by genetic variation.

The animal model is a mixed linear effects model used to partition phenotypic variance into sources of non-independence, such as shared genotypes or shared environment. This statistical tool is a powerful way of estimating heritability, as it allows for additional components to be estimated (Kruuk 2004; Kruuk & Hadfield 2007; Wilson et al. 2010). I used a Bayesian statistical approach with Markov Chain Monte Carlo sampling (Hadfield 2010); the advantage of this approach over a maximum likelihood one is that uncertainty around estimates carries over with subsequent calculations. Therefore, I was able to estimate heritability and various environmental effects *and* the uncertainty around those estimates.

In altricial nestlings, genetic and environmental variation are confounded as related individuals also share the same environment. Therefore, as detailed in chapter 4, I performed a cross-fostering experiment to decouple these genetic and environmental influences. In this experiment, I exchanged two chicks between nests of equal age and brood size at two days post hatching. As a result of this experimental design, I was able to estimate phenotypic variation explained by additive genetic variation, the early nest environment, and the rearing nest environment. The early nest environment includes incubation effects and any parental effects that occur within 48 hours of hatching. The rearing environment includes micro-climate effects and parental effects for the remainder of the nestling period. I found that again, genetic variation explained the least phenotypic variation (approximately 16%), although this component is still strong enough to elicit an evolutionary response to selection. As I found in chapter 3, environmental variation (both early and rearing environment) explained a large proportion of phenotypic variation (collectively 70%), suggesting that the early environment is important for development of both juvenile and subsequent adult plumage color. Given this finding, I can infer whether coloration is used in mate choice to gain direct benefits (including high quality habitat,

and parental care) or indirect benefits (good genes). My results suggest that females primarily gain information about a mate's developmental environment, which may be indicative of other aspects of quality. They also gain indirect benefits, however, as their offspring will gain alleles for dark plumage from a dark father. This may explain the high rate of extra-pair paternity in Colorado.

In chapter 5, I compare the genetic variance/covariance structure (G) of plumage coloration between populations from North America and the Czech Republic. I replicated the cross-fostering experiment and found that the proportion of total phenotypic variation explained by genetic and environmental variation was similar to the Colorado population. However, using comparative quantitative genetic methods, I found that G differs between these populations, indicating that similar selection on color will produce different evolutionary responses in these populations. Moreover, given the evidence that sexual selection differs among these populations, it is possible that past divergent selection has produced the existing phenotypic differences.

Collectively, this dissertation provides insight into the environmental effects on continuous melanin-based coloration, a trait that is typically considered to be under strong genetic control. Using this information, I offer testable hypotheses for why females use ventral coloration when making mate choice decisions, thus making a connection between mechanism and function. Additionally, I provided a framework for how quantitative genetic analyses can be used to draw conclusions about mechanisms of sexual selection (function) based on the relative influences of genetic vs. environmental variation (mechanism). By comparing genetic variance/covariance matrices between populations, I have provided evidence of differences in genetic variation between phenotypically divergent populations of barn swallows. This information can be used to infer how selection will affect additional phenotypic divergence

between these populations. Along with existing evidence for divergent selection, I also argue that past selection, rather than drift, has driven the existing phenotypic divergence. I hope this work will provide a foundation for future comparative quantitative genetic research on sexual signals to better understand their role in phenotypic divergence and speciation.

Chapter 2

Vertebrate Pigmentation: From Underlying Genes to Adaptive Function¹

2.1 Abstract

Animal coloration is a powerful model for studying the genetic mechanisms that determine phenotype. Genetic crosses of laboratory mice have provided extensive information regarding patterns of inheritance and pleiotropic effects of loci involved in pigmentation. Recently, the study of pigmentation genes and their functions has extended into wild populations, providing additional evidence that pigment gene function is largely conserved across disparate vertebrate taxa and can influence adaptive coloration, often in predictable ways. These new and integrative studies, along with those using a genetic approach to understand color perception, raise some important questions. Most notably, how does selection shape both phenotypic *and* genetic variation, and how can we use this information to further understand the phenotypic diversity generated by evolutionary processes?

¹ Published as: Hubbard, JK, Uy, JAC, Hauber, ME, Hoekstra, HE, and Safran RJ (2010) *Trends in Genetics*, **26** (3) 231-239.

2.2 Genotypes and Phenotypes

A fundamental pursuit in the field of evolutionary genetics is to determine the underlying molecular mechanisms that lead to natural variation in morphology, physiology and behavior (an individual's 'phenotype'). Understanding the link between genotype and phenotype can elucidate mechanisms that shape phenotypic variation within populations and how these affect patterns of evolutionary change. For example, knowing the underlying genetics of traits can reveal the type of evolutionary change affecting phenotypic variation (Barrett et al. 2008) as well as the strength and timing of selection (Linnen et al. 2009). Thus, identifying the mechanisms that shape variation in morphology and behavior can offer important insights into the process of population divergence and speciation.

The study of mammalian pigmentation has long served as a model system to learn about molecular, cellular and developmental processes (Silvers 1979). As a result, over 150 genes that affect animal color and patterning have been identified (Roulin 2004; Mundy 2005; Hoekstra 2006; Protas & Patel 2008). Although most of these genes were first identified in laboratory mice (genus *Mus*), they have more recently been examined in domestic and natural populations (Theron et al. 2001; Mundy et al. 2004; Hoekstra et al. 2004; Doucet et al. 2004; Steiner et al. 2007; Anderson et al. 2009; Uy et al. 2009), and thus are relevant to understanding the underlying molecular basis of adaptation in the wild. Dissection of the genetic architecture responsible for color variation in nature affords opportunities to ask questions about i) how selection on specific parts of the genome influences phenotype (mechanism), and in turn, ii) how selection on phenotype itself (function) affects these genomic regions known to underlie various aspects of pigmentation. Still, these are early days in understanding the connections between the mechanistic and functional basis of animal coloration. In this review, we build on what is already

known about the genetic basis and the developmental mechanisms generating the diversity of pigmentation and color patterns in vertebrates (Roulin 2004; Mundy 2005; Hoekstra 2006; Protas & Patel 2008), and highlight the importance of making new, explicit links between selection on both genotype and its associated phenotype in order to gain a comprehensive view of how the interaction and feedback of genetic and phenotypic variation are simultaneously shaped by evolutionary processes.

2.3 Adaptive Function of Coloration

In animals, coloration, via both pigmentation and nanostructure, has many functions. For example, coloration is often used for intraspecific communication – (e.g., ornamental color used for mate choice and intrasexual competition (Safran & McGraw 2004; Amundsen & Pärn 2006; Hill 2006; Senar 2006; Protas & Patel 2008)) and interspecific interactions (e.g., aposematic and cryptic coloration used for predator avoidance (Slagsvold et al. 1995; Amundsen & Pärn 2006; Protas & Patel 2008)). In many rodent species, coat color (i.e., pelage) closely matches the local substrate to minimize detection by visually-hunting predators (Nachman et al. 2003; Steiner et al. 2009; Linnen et al. 2009). Moreover, many colors and pigments can have other adaptive functions such as photoprotection (McGraw 2006a, 2006b; Protas & Patel 2008), structural support (McGraw 2006a), microbial resistance (Goldstein et al. 2004), and thermoregulation (Rosenblum et al. 2004; McGraw 2006a; Protas & Patel 2008). Because in most cases animal color is likely influenced both by genetic and environmental (e.g., nutritional status, maternal effects, disease state) factors it is instructive to isolate the genetic component of color traits to i) predict the amount of selection required for an evolutionary response in these traits; ii) determine the degree to which parental phenotype predicts offspring phenotype, or how

heritable the trait is; and iii) better understand the proximate mechanisms driving or constraining evolutionary processes. A critical consideration for the function of coloration traits with putative signaling roles is the visual perception of the receiver (see below). Indeed, measurable phenotypic differences are only biologically meaningful if the phenotypic change is detectable by the receiver.

2.4 Pigmentation Genes Involved in Melanin-based Coloration

For melanin-based coloration, an impressive number of pigmentation genes have been identified, cloned and sequenced in laboratory mice (Hoekstra 2006). These genes are scattered throughout the genome and are involved in a variety of cellular processes (Hoekstra 2006). Despite the large number of potential targets, only a handful of genes have been identified as major contributors to color variation in a wide array of animal taxa. Of these, the melanocortin-1 receptor (*MC1R*) and agouti signaling protein (*ASIP*), both important in melanin synthesis (Hoekstra 2006), are among the most widely studied pigmentation genes in wild populations of mammals, birds, reptiles, and fish (see Fig. 2.1) (e.g., Theron et al. 2001; Rosenblum et al. 2004; Gross et al. 2009; Kingsley et al. 2009). The majority of these studies have concentrated on uncovering the genetic basis of intraspecific differences between populations with discrete polymorphisms (e.g., light and dark colored mice) (Mundy et al. 2004; Rosenblum et al. 2004; Hoekstra et al. 2006; Steiner et al. 2007; Uy et al. 2009). The wealth of knowledge about the molecular mechanisms underlying melanin-based coloration is unmatched relative to current information regarding carotenoid-based or structural coloration. It is worth noting, however, that structural coloration is likely influenced by melanin pigmentation genes because in birds, reptiles, and fish, the underlying basis of structural colors often involves melanin pigments

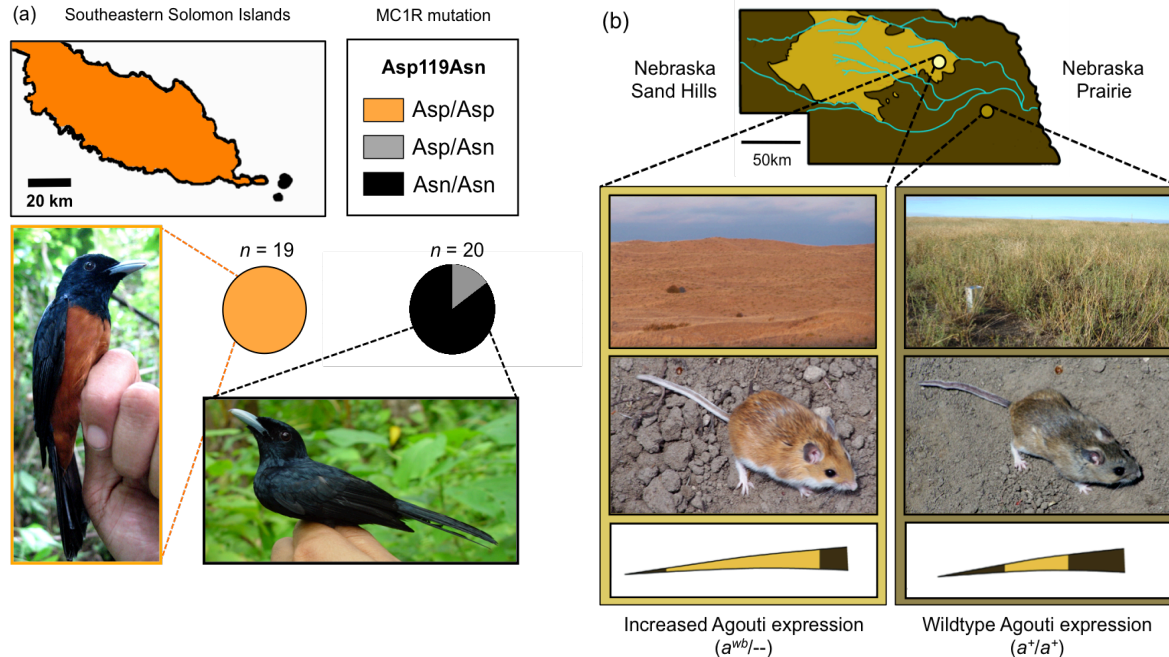


Figure 2.1. Association between mutations in pigmentation genes and color variation in natural populations of vertebrates. Mutations in *MC1R* and *ASIP* can have large effects on vertebrate coloration, which can be important in the origin of new species or local adaptation within species. **(A)** *Monarcha castaneiventris* flycatchers show distinct variation in plumage color throughout the Solomon Islands and may represent the early stages of species formation (Uy et al. 2009). Distribution, plumage color and *MC1R* genotype frequency (pie charts) of the chestnut-bellied and melanic flycatchers of southeastern Solomon Islands are shown. Ranges of the two subspecies are given: orange, chestnut-bellied form (*M. c. megarhynchus*; Makira Island) and black, melanic form (*M. c. ugiensis*; Santa Ana and Santa Catalina). A single *MC1R* amino-acid substitution is perfectly associated with color variation important for species recognition, linking this mutation with the early stages of speciation. **(B)** Driven by selection for crypsis from visual predators, deer mice (*Peromyscus maniculatus*) have evolved pelage to match their local substrate (Linnen et al. 2009). Location, habitat and hair banding pattern of deer mice living on and off of Nebraska's Sand Hills are shown. Both mice are pictured on dark-soil background: yellow, *P. m. luteus* and brown, *P. m. bairdii*. *Cis*-acting mutation(s) at the *Agouti* locus are associated with changes in *Asip* expression and width of the subapical phaeomelanin hair band, leading to overall differences in coat-color brightness and ultimately survival. The dominant *agouti wideband* (a^{wb}) and recessive wildtype (a^+) phenotypes/alleles are pictured.

(Prum 2006). By contrast, carotenoid coloration is likely to be under less genetic control than melanin-based coloration because these molecules are derived from diet (McGraw 2006b), rather than being synthesized endogenously (McGraw 2006a). Consequently, there is still much to learn about the proximate mechanisms that control the dazzling array of colorful phenotypes which are

often the target of both natural and sexual selection, and also are known to play a role in defining boundaries among populations and species (Seehausen et al. 2008; Uy et al. 2009).

2.4.1 Melanocortin-1 Receptor.

MC1R is a seven-transmembrane domain G protein-coupled receptor (GPCR) found primarily in melanocytes which acts as a switch to control the type of melanin synthesized for deposition in tissues (Mountjoy et al. 1992). In mammals and birds, the ratio of eumelanin and pheomelanin largely determines an animal's overall color: darker (black to brown) phenotypes result from the increased deposition of eumelanin, and lighter (red to yellow) phenotypes result from increased deposition of pheomelanin (McGraw et al. 2004, 2005; McGraw 2006a).

Whereas melanocyte stimulating hormone (α -MSH)-mediated *MC1R* activation induces eumelanin production, *ASIP* antagonizes *MC1R* and triggers pheomelanin production. Lizards and fishes, by contrast, do not produce pheomelanin (Rosenblum et al. 2004), and in these taxa, *MC1R* likely affects eumelanin density, rather than melanin type.

MC1R is highly conserved among vertebrates and has a relatively simple genetic structure (single 1-kb exon), which has facilitated its identification in a diversity of taxa. As a result, dozens of studies now show a link between variation in *MC1R* and pigmentation in numerous vertebrates (Roulin 2004; Mundy 2005; Hoekstra 2006; Protas & Patel 2008; Mills & Patterson 2009), but see refs (Rosenblum et al. 2004; Cheviron et al. 2006; Steiner et al. 2009) for examples where melanin color does not associate with *MC1R* variants. The majority of these studies have statistically associated a single-nucleotide polymorphism (SNP) and the resulting amino acid change, with a discrete color polymorphism. Known mutations are largely interspersed throughout the protein-coding sequence, yet distinct mutations in closely related species as well as identical mutations at homologous positions in diverse taxa can lead to the

same or similar phenotypes (Table 2.1; Appendix 1). For example, the Arg⁶⁵Cys substitution contributes to pale coloration in beach mice (*Peromyscus polionotus*) that inhabit Florida's sandy coast (Hoekstra et al. 2006); the identical mutation is found in woolly mammoths (*Mammuthus primigenius*) (see Appendix 1) (Römpler et al. 2006). *In vitro* assays (see section 2.6) done in both species demonstrate that this single mutation causes a decrease in receptor signaling by reducing ligand binding (Hoekstra et al. 2006), suggesting that like beach mice, mammoths also might have varied in coat color (Römpler et al. 2006). Importantly, as done here, statistical

Table 2.1. Pigmentation genes associated with color variation in wild populations of vertebrates.

GENE	DERIVED PHENOTYPE	CLASS	KEY REFS
<i>MC1R</i> ^A	Darker Skin, Plumage, Coat	Actinopterygii, Aves, Mammalia	(Theron et al. 2001; Nachman et al. 2003; Mundy et al. 2004; Römpler et al. 2006; Gross et al. 2009)
	Lighter Skin, Coat	Mammalia, Reptilia	(Ritland et al. 2001; Rosenblum et al. 2004; Hoekstra et al. 2006; Lalueza-Fox et al. 2007)
<i>ASIP</i> ^A	Darker Coat	Mammalia	(Våge et al. 1997; Kingsley et al. 2009)
	Lighter Coat	Mammalia	(Linnen et al. 2009)
<i>TYRP1</i> ^A	Darker Plumage	Aves	Laura Buggiotti, PhD Thesis, University of Turku, 2007
	Lighter Coat	Mammalia	(Gratten et al. 2007)
<i>OCA2</i>	Lighter Skin	Actinopterygii	(Protas et al. 2006)
<i>K locus</i> ^B	Darker Coat	Mammalia	(Anderson et al. 2009)
<i>KITLG</i>	Lighter Gills, Skin	Actinopterygii	(Miller et al. 2007)
<i>SLC24a5</i>	Lighter Skin	Actinopterygii	(Lamason et al. 2005)
<i>Pax7</i>	Dark Skin Blotches	Actinopterygii	(Roberts et al. 2009)

^A Mutations/genetic variants have been identified in this gene that associate with parallel phenotypic changes in lab populations and/or domestic animals

^B Mutations/genetic variants have *not* been identified or shown to have an affect on human skin, hair, or eye color (see Sturm 2009).

associations between *MC1R* mutations and color should be functionally verified as sometimes even mutations strongly associated with color variation have no measurable effect on receptor function (section 2.6) (Steiner et al. 2009; Rosenblum et al. 2010). For *MC1R* this can be achieved via cell-based pharmacology assays (Römpler et al. 2006; Lalueza-Fox et al. 2007), although transgenic assays remain the ‘gold standard.’

The number of studies that have implicated *MC1R* amino acid changes in color evolution, as well as the diversity of organisms in which these changes have been identified, is intriguing and raises the question of why *MC1R* repeatedly appears to affect vertebrate coloration. Potential answers to this question include minimal pleiotropic effects of *MC1R*, large mutational target size, high mutation rate, and ascertainment bias due to its simple and conserved structure (see Mundy 2005; Protas & Patel 2008; Fang et al. 2009).

2.4.2 Agouti Signaling Protein (Agouti or ASIP).

ASIP is a paracrine signaling protein antagonist of *MC1R* that causes melanocytes to switch from producing eumelanin to pheomelanin. Multiple *ASIP* mutations are associated with color change (e.g., Våge et al. 1997; Mundy & Kelly 2006; Nadeau et al. 2008; Kingsley et al. 2009); however, compared to *MC1R*, the number of examples from wild populations is far fewer, and the types of molecular changes associated with color are different. Whereas all known *MC1R* mutations occur within the coding region, the genetic changes in *ASIP* occur both in the coding (Mundy & Kelly 2006) and regulatory regions (Klovins & Schiöth 2005; Nadeau et al. 2008; Kingsley et al. 2009). While *ASIP* has been primarily studied in mammals, it appears to affect color in a variety of species including wild rodents (Steiner et al. 2007; Kingsley et al. 2009; Linnen et al. 2009), domestic horses (*Equus ferus*) (Ludwig et al. 2009), domestic cats (*Felis domesticus*) (Eizirik et al. 2003), and foxes (*Vulpes vulpes*) (Våge et al. 1997). *ASIP* has

also been studied in fishes (Cerdá-Reverter et al. 2005; Klovins & Schiöth 2005) and birds (Klovins & Schiöth 2005; Nadeau et al. 2008). To our knowledge, however, Agouti-like sequences have not been reported in reptiles.

Mutations in *ASIP* that are associated with color differences typically affect *ASIP* expression. For example, variation in *ASIP* mRNA expression levels are often highly correlated with pigmentation (Steiner et al. 2007; Kingsley et al. 2009). Increased expression, including experimental over-expression, of *ASIP* increases phaeomelanin production due to its antagonistic effect on MC1R. In rodents this can lead to an increased phaeomelanin band on individual hairs as seen in mice inhabiting the light-colored substrate of Nebraska's Sand Hills (Fig. 2.1) (Linnen et al. 2009) or at the extreme, a completely blonde mouse (Hoekstra 2006). By contrast, loss-of-function mutations tend to cause the exclusive production of eumelanin and a melanic coat-color phenotype (e.g., Kingsley et al. 2009). Although mutations in *ASIP* can affect melanin production and are associated with coloration, functional studies in wild populations remain largely absent.

Unlike *MC1R*, *ASIP* has well-described pleiotropic effects. In lab mice, the classic obese *yellow* mutant is the result of *Asip* overexpression in hair follicles leading to light color and also the misexpression of *Asip* in the brain where it interacts with the melanocortin-4 receptor (MC4R) and causes a re-feeding behaviour and ultimately obesity (Fan et al. 1997). Moreover, this *yellow* mutation, when homozygous, is lethal (Nadeau et al. 2008). In Japanese quail (*Coturnix japonica*), the *yellow* mutation, which also causes *ASIP* upregulation, resides in a similar genomic position as the *lethal yellow* mutation in mouse (Nadeau et al. 2008). As in mice, when homozygous, the Japanese quail mutation is lethal, whereas heterozygotes have wheat-straw yellow-colored feathers (Minvielle et al. 2007). Nadeau et al (Nadeau et al. 2008)

argue that these similarities suggest that *ASIP* expression pattern and function is conserved across vertebrates (Jackson 1997; Jackson et al. 2006). Along with its complex gene structure and challenges associated with identifying regulatory mutations, its pleiotropic effects might explain why few associations between color and genetic variation at *ASIP* have been reported.

In addition to their independent effects, there are well-characterized epistatic interactions between *MC1R* and *ASIP*. In laboratory mice *Mc1r* is epistatic to *Asip*; for example, dominant mutations in *Mc1r* that lead to a constitutively active receptor are not inhibited by *Asip* (Ollmann et al. 1998). However, in foxes, *ASIP* can counteract a constitutively active *MC1R* (Våge et al. 1997). Another unique interaction has been found in beach mice: *Mc1r* mutations that lead to lighter coloration are only visible when a mutation leading to increased *Asip* expression is also present (Steiner et al. 2007). These studies highlight that the phenotypic effects of both *MC1R* and *ASIP* mutations can be highly dependent on the genetic background in which they arise, and more generally, that interaction effects are allele- (not gene-) specific and thus likely to vary among populations and species.

2.4.3 Other pigmentation genes.

Recently, a growing number of new studies in both domestic and wild animals have shown that several pigmentation genes originally identified in laboratory mice also play important roles in determining color variation in domestic and natural populations of vertebrates. For example, sequence variants of tyrosinase-related protein 1 (*TYRPI*), which codes for a melanogenic enzyme involved in production of eumelanin (Zdarsky et al. 1990), have been associated with color variation in several domestic animals including dogs, cats, and cattle, as well as lab populations of Japanese quail (Nadeau et al. 2008). Additionally, a single SNP in

TYRPI is associated with a color polymorphism in wild Soay sheep (*Ovis aries*) (Gratten et al. 2007), and transcript variants possibly explain color variation between wild populations of *Ficedula* flycatchers (Laura Buggiotti, PhD Thesis, University of Turku, 2007). Moreover, tyrosinase (*Tyr*) knockouts cause albinism in lab mice (Kwon et al. 1989), whereas albinism in cave dwelling Mexican tetra (*Astyanax mexicanus*) is associated with multiple, independently derived polymorphisms in ocular albinism type 2 (*OCA2*) (Protas et al. 2006), a gene known to determine iris color in humans (Sturm & Frudakis 2004). Melanism in the gray wolf (*C. lupus*) showed no association with *MC1R* or *ASIP* mutations, but rather with a different member of the melanocortin pathway, the *K* locus (Anderson et al. 2009). A novel allele at the *K* locus appears to have been introduced via introgression from domestic dogs, as the same 3-base pair deletion is associated with dark coats in dogs, coyotes and wolves (Anderson et al. 2009). Finally, a recent and intriguing study implicates *cis*-regulatory changes in a highly conserved developmental gene, *Pax7*, in the orange-blotch (OB) phenotype in cichlid fish of Lake Malawi (Roberts et al. 2009). This OB allele might be the target of sexually antagonist selection, that is, it provides a camouflaging phenotype to females but disrupts species-specific male coloration important in mate selection.

Two additional studies have demonstrated that pigmentation genes identified in fishes may also influence human skin color. First, differences in mRNA expression levels of Kit ligand (*KITLG*) are associated with changes in gill and skin coloration in stickleback fish (*Gasterosteus aculeatus*) (Miller et al. 2007). *KITLG* controls the proliferation, migration, differentiation, and survival of Kit receptor-expressing melanocytes and therefore melanin patterning (Wehrle-Haller 2003). The same study implicates a *cis*-regulatory change in *KITLG* in human skin coloration as patterns of nucleotide polymorphism are consistent with selection in human populations with

different skin phenotypes (Miller et al. 2007). Second, the *golden* mutation in zebrafish (*Danio rerio*) was linked to a diminished number, size and density of melanosomes, and ultimately to a mutation in *SLC24a5*, a putative potassium-dependent sodium/calcium exchanger (Lamason et al. 2005). In humans, an ancestral *SLC24a5* allele predominates in African and East-Asian populations, but a derived allele, defined by a coding mutation, is nearly fixed in European populations; the derived allele also is associated with light skin color in admixed populations (Lamason et al. 2005). Because mice show little variation in skin color, fishes or other taxa with known variability of epidermal pigmentation (e.g., Lamason et al. 2005; Miller et al. 2007; Eriksson et al. 2008) are more promising models for studying human skin pigmentation.

One striking observation is that many of these pigment genes affect production of eumelanin, or the switch between production of eumelanin and pheomelanin (as with *MC1R* and *ASIP*). By comparison, we know very little about genes that are involved strictly in the synthesis of pheomelanin, or in other steps of melanogenesis, e.g. ways in which pigment density or concentration are controlled. We expect that future studies that genetically dissect the mechanisms that control different aspects of the melanin pathway will be especially useful for understanding the proximate mechanisms responsible for more subtle variation in color, for example, continuous variation within species that could be an important target of local adaptation and mate choice.

2.5 Pigmentation Genes Involved in Non-melanin-based Coloration

In addition to melanin pigments, animal coloration can involve nanostructure of the tissue, carotenoid pigments and a handful of other pigments (e.g., pterins found in parrots and lizards (McGraw 2006c; Steffen & McGraw 2007)). To date there is very little known about the

genetic mechanisms that underlie coloration caused by structure or non-melanin pigments. A recent study of domestic chicken (*Gallus gallus domesticus*) showed that variation in expression levels of beta-carotene dioxygenase 2 (*BCD02*), a gene involved in cleaving β -carotene to create colorless apocarotenoids, is strongly associated with yellow versus white skin (Eriksson et al. 2008). With few exceptions, most of what is known about the genetic basis of carotenoid-based traits comes from studies of heritability rather than of specific genes. Some heritability estimates of carotenoid-based plumage suggest strong genetic effects (e.g., $h^2 = 0.84$ in house finches (Hill 1991), and in fish (Hughes et al. 2005)). However, often these studies fail to control for environmental influences on color, as the brightness and hue of carotenoid-based traits are tightly linked to availability of dietary carotenoids (see section 2.7) and can be quite condition dependent. Accordingly, most studies of carotenoid-based coloration report low heritability, as demonstrated by a study in blue tits (*Cyanistes caeruleus*) (Hadfield & Owens 2006). Yet, there are many steps along the biosynthetic pathway leading to tissue deposition where genetic variation could have an effect (e.g., absorption from food, transport, sequestration, esterification) (McGraw 2006b). Consequently, identifying individual genes, or classes of genes, that affect carotenoid-based coloration appears to be a daunting task, but one that should produce high rewards.

Similar to carotenoid-based coloration, insights into the genetic basis of structural coloration have been limited mostly to heritability estimates and condition dependence in birds and fish (Kelsh 2004; Siefferman & Hill 2005; Basolo 2006; Hadfield & Owens 2006). Structural colors, excluding white, have a base layer of pigment to absorb light and prevent incoherent scattering by the underlying tissue (Prum 2006); in birds, this pigment layer is usually melanin, however there are also examples of carotenoid pigment base layers (Prum 2006).

Consequently, the same genes that affect the underlying pigments likely affect structural color traits. Yet, to our knowledge, no study has explored the role of known melanin pigmentation genes on structurally-based color traits (but see Uy et al. 2009). Because the nanostructure of the tissue (e.g., feathers, skin, hair) determines how light scatters within the tissue (Prum 2006), the developmental mechanisms that control nanostructure (Prum et al. 2009) are also potential targets of selection and may be a treasure trove for genetic influences on structural color.

2.6 Establishing causal relationships between mutation and phenotypic change

MC1R can be expressed *in vitro* and assayed for membrane integration, ligand binding and cyclic AMP activation (Mundy 2005). A recent study in lizards highlights both the importance of functional studies and ways in which different functional mechanisms can produce similar changes in color. Three lizard species (*Sceloporus undulatus*, *Aspidoscelis inornata*, and *Holbrookia maculata*) colonized the 8000 year-old White Sands in New Mexico, and each has evolved a similar blanched phenotype relative to their darker ancestors that inhabit the surrounding desert. All three species each have a single coding mutation in *MC1R* that is statistically correlated with phenotype (Rosenblum et al. 2004), but when functionally assayed, *MC1R* alleles from each species produced different results (Rosenblum et al. 2010). In *H. maculata*, there was no measurable difference in receptor activity; in *A. inornata*, the mutation resulted in lowered signaling potential; and in *S. undulatus*, the derived mutation decreased the efficiency by which MC1R integrated into the melanocyte membrane. Thus, it is clear that different *MC1R* variants can result in similar phenotypes but through different functional mechanisms (Rosenblum et al. 2010).

MC1R mutations can also be functionally verified using other methods. For example, mutations identified in Mexican tetra populations were assessed using the model organism, zebrafish. Gross et al. (Gross et al. 2009) first demonstrated that knocking out *MC1R* in zebrafish resulted in a qualitatively lighter phenotype. Next, using Mexican tetra RNA transcripts from both surface and cave populations, they showed that the surface transcript rescued the ancestral phenotype whereas the transcript from the cave populations did not. A causative link between *OCA2* variants and pigmentation differences between surface and cave populations of Mexican tetra was established using similar phenotype rescue experiments (Protas et al. 2006). These heterologous experiments (either cell-culture based or *in vivo* assays) provide convincing evidence that the mutations found in the respective pigmentation genes indeed cause the observed phenotypic changes.

2.7 Environmental influences on color

Color is not a physical phenomenon; rather, it is the perceptual image formed by the sensory filters and the cognitive architecture of the observer. As such, color, like many other perceptual phenomena, is extremely malleable and dependent on environmental context (Endler & Thery 1996). Take for example the rainforest dwelling eclectus parrot (*Eclectus roratus*) whose bright crimson and navy females sharply contrast with the duller monochromatic green males when judged in captivity by the human eye, leading this species to be classed as an example of “reversed sexual dimorphism” (Heinsohn 2008). However, in nature, males are less conspicuous against the background foliage than females when viewed by their avian predators, which likely confers a selective advantage because males forage and provision females almost exclusively during their prolonged breeding season. In turn, both females and males benefit from

being more conspicuous against tree trunks than foraging males when viewed by the parrot visual system – females display to other females in competition for scarce nesting cavities and males display at cavities to females for mate attraction (Heinsohn et al. 2005). The environmental context of perceived coloration can thus be dependent on both the micro- and macro- habitat in which individuals display, or on the circadian and seasonal variation in ambient light sources and filters (e.g., cloud coverage, substrate color, canopy structure, and foliage coloration), including natural or anthropogenic change in visibility and turbidity (Endler 1993; Nachman et al. 2003).

In addition, temporal and geographic differences in the local availability of chemical and energetic resources necessary for the collection, transport, biosynthesis, incorporation, and behavioral display of pigmentation patterns can also result in variation of color displays and physical function. For example, long-term pedigree data revealed that eggshell maculation patterns are inherited through female sex-specific genetic elements in great tits (*Parus major*) breeding in Wytham Woods near Oxford (Gosler et al. 2000). However, in nearby populations, the reduced availability of environmental calcium is correlated with increased density of the protoporphyrin-containing speckles concentrated in the thinner zones of the eggshell matrix, likely serving to increase structural strength of the eggs in calcium-poor habitats (Gosler et al. 2005). Thus, environmental factors clearly can influence the appearance of eggshells. Finally, rapid, physiological modulation of an individual's coloration for crypsis, mimicry, or sexual display –like the incredible ability of cuttlefish to change from cryptic to showy coloration in the blink of an eye – illustrates the potential scope of diverse adaptive functions of dynamic feedback between coloration, sociality, and the environment (Safran et al. 2008a; Rubenstein & Hauber 2008).

2.8 Linking mechanism and function

Using model organisms, we have gained great insight into the underlying genetic basis of pigmentation, specifically melanin-based pigmentation. With advancing technology, it is now possible to study molecular mechanisms of pigmentation in non-model, and even wild, systems. Indeed, these studies have demonstrated highly conserved function of many of these genes across species. These recent genotype-phenotype associations also can inform our understanding of the evolutionary process leading to adaptive coloration. For example, we would like to know i) how many genes affect pigment variation in natural populations? ii) How often are the same genes involved in convergent phenotypes? iii) How does the strength of selection affect color variation? and iv) Can we detect evidence of selection in patterns of nucleotide variation in pigmentation genes? We are just now beginning to understand the genetics underlying adaptive changes in coloration and color vision (see section 2.9), and in cases when these differences influence reproductive isolation, we also might be able to make inferences about the genetics of speciation.

To address these questions, we need a deep understanding, at the molecular, genetic and developmental level, of how changes in pigmentation genes and their interactions produce changes in color phenotype. Studies that have reported perfect associations between *MC1R* variants and coloration (see Hoekstra et al. 2004; Doucet et al. 2004; Mundy 2005; Uy et al. 2009) provide convincing evidence that, in some cases, single genes can be responsible for phenotypic change, especially in cases where no intermediate phenotypes are found and Mendelian inheritance is clear. However, few studies have explored the role of more than one pigmentation gene in determining phenotype (but see Steiner et al. 2007). Interestingly, most studies that have explicitly looked at more than one gene have found that interactions between

genes affect phenotype (Våge et al. 1997; Steiner et al. 2007; Nadeau et al. 2008). Consequently, it remains difficult to determine precisely how many genes underlie color change.

Current evidence from pigmentation genetics in laboratory, domesticated and wild populations show that many genes are involved in pigmentation (Appendix 1). There are many examples in which the same genes (e.g. *MC1R*, and to a lesser extent *ASIP*) are repeated targets of evolutionary change. On the other hand, different pigmentation genes and/or different functional mechanisms in the same gene (e.g., Theron et al. 2001; Hoekstra & Nachman 2003; Uy et al. 2009) can produce very similar phenotypes even among populations within a species (Table 2.1) (e.g., Hoekstra et al. 2006; Römler et al. 2006). This suggests some genetic and developmental constraints, and at the same time, flexibility in the underlying mechanisms of adaptation.

Color traits are often the target of selection, as in many cases even small changes in color can have large implications for an organism's ability to survive or reproduce in the wild (e.g., Endler 1991; Safran & McGraw 2004). Although field observations and experiments can provide estimates of the strength of selection (e.g., Grant 1985; Hoekstra et al. 2004), the identification of genes underlying adaptive traits allows us to estimate selection at the genetic level. For example, assuming a model of migration-selection balance at equilibrium (Haldane 1930), selection coefficients can be estimated directly based on estimates of effective population size and migration rate from (neutral) genetic data. This approach was used to estimate strong selection against 'mis-matched' mice – selection against the ancestral light color morph on novel dark soil habitat as well as the derived dark morph on light habitat (Hoekstra et al. 2004). In addition, selection coefficients can be estimated using more sophisticated population-genetic approaches based on patterns of nucleotide variation (reviewed in Nielsen 2005; Biswas & Akey 2006;

Jensen et al. 2007). For example, several methods take advantage of linkage disequilibrium (LD), or the association among mutations from independent loci, to detect a signature of selection. The extent of LD should increase in regions under strong directional selection, and so genomic regions surrounding the target of selection initially will have high LD and low polymorphism (i.e., selective sweep) (Kim & Stephan 2002; Meiklejohn et al. 2004). This method was used effectively in detecting and estimating the strength of selection on *Asip* allelic variants in *P. maniculatus* (Linnen et al. 2009). For more in-depth discussions of the various analytical techniques developed to detect selection at the molecular level several recent reviews are available on this topic (Nielsen 2005; Biswas & Akey 2006; Jensen et al. 2007).

Thus, it is clear that identifying the genetic basis of phenotypic traits can provide insight into the evolutionary process. Owing to the relative success of linking genotype to phenotype for pigmentation traits, much of this progress has come from the study of color variation in vertebrates. Future work, which will involve studies in diverse taxa and unique color variants, including brilliant colors, more complex color patterns, and continuous color variation, will only increase our growing knowledge of the molecular basis of organismal phenotypic diversity. In addition to identifying informative genetic mutations underlying adaptive coloration in wild populations of vertebrates, future studies should quantify selection at the phenotypic and molecular level to make progress toward understanding the evolutionary processes leading to phenotypic change.

2.9 Studying the genetic basis of avian color perception

Physical measures of coloration based on reflectance spectrometry have revolutionized the field of color research, compared to earlier work that relied on human-assessed metrics

(Cuthill et al. 1999). Yet, when the putative function of color diversity is signaling, it is also critical to identify what color differences are perceptible to the intended receiver (Cassey et al. 2009). To do so, measures of light reflectance must be filtered through the sensory range and perceptual thresholds of the recipient (Endler et al. 2005). Sensory neurophysiology and behavioral psychophysics can identify both the range and the error in perceiving and responding to color differences, but these methodologies are not always suitable for large scale evolutionary studies, or even for species-specific studies on subjects that are intolerant of captivity (Osorio & Vorobyev 2008).

In birds, the functional interpretation of diverse avian plumage and egg coloration has benefited from large-scale comparative approaches. For example, recent studies have focused on the most variable component of avian color sensitivity – the violet or ultraviolet receptor sensitivity of the opsin gene (Shi & Yokoyama 2003). Specifically, DNA sequencing of an individual's short-wavelength opsin receptor (SWS1) can provide information about its function. Functional differences can be measured based on the known peak absorbance of opsin types previously isolated or, in the case of novel sequences, via *in vitro* mutagenesis and functional tests of light absorbance (Ödeen et al. 2009). Using non-invasive genetic means to measure visual perception in avian color communication is especially relevant for understanding the functional and ecological context of avian color variation. For example, the frequent mismatch between the ultra-violet sensitivity of hosts and their violet-sensitive egg-mimetic brood parasites, or between tetrachromatic avian prey and their dichromatic mammalian predators, enables the evolution of private communication channels protected from the risks of “eavesdropping” (Göth & Evans 2004).

Similar approaches that combine knowledge of opsin protein sequences and their respective functions have broad applications for many vertebrate color-vision studies. For example, within a sympatric species flock of Lake Victoria cichlids, expressed opsin sequences and *in vitro* predictions of their respective peak sensitivities tightly correlate with depth range (which modulates illumination spectra), carotenoid-based male polychromatism, and behavioral measures of female choice (Seehausen et al. 2008). Ultimately, we would like to alter opsin genes and measure resultant changes in visual ability. Recently, progress toward this goal was made when viral delivery of the human version of red-sensitive opsin led the otherwise dichromatic male squirrel monkeys to “catch up” with trichromatic female conspecifics’ abilities in color discrimination (Mancuso et al. 2009).

2.10 Outstanding Questions

Here we offer questions at the interface of pigmentation and vision genetics and their ecological and evolutionary context.

- (1) How often do species with variation in color also show variation in color perception?
- (2) Are genes underlying the coloration of fur, skin, scales, feathers, and eggshells linked to (either physically or statistically), and co-evolving with, genes underlying perceptual bias in color vision (e.g., opsin genes)?
- (3) Which evolved first: genes responsible for changes in pigmentation or those related to visual perception? Can we use phylogenetic comparative methods to date evolutionary origins of variation in pigmentation and perception?
- (4) Which genes (pigmentation or opsin) are less constrained for local adaptation (e.g., via co-evolutionary arms races with predators or as light environment changes)?

- (5) How often are genotypes underlying within-population and among-population color polymorphism (fur, skin, scales, and feathers) maintained by non-random mating patterns?
- (6) Are pigmentation or opsin genes more often associated with coloration differences among closely related populations?

2.11 Concluding Remarks

Data on *MC1R* and *ASIP* have accumulated at a rapid rate, and offer some of the first direct links between ecologically relevant phenotypes and their underlying genotypes. Yet, there is much work to be done, even with these genes. First, we emphasize the need for careful functional assays not only to demonstrate empirically the causal links between genotype and phenotype, but also to provide a more detailed understanding of *how* mutations produce phenotypic variation (e.g., mechanism). Second, using population-genetic approaches and/or experimental field studies we can also document selection at both the genetic and phenotypic levels (e.g., function). Of course, from a comparative perspective, future work will expand the scope of chemical, structural, and genetic analyses to understand the mechanisms generating the awe-inspiring array of animal coloration, not only color variation controlled by well-characterized melanin-related genes, but also from the brilliant plumages, scales, skin, and other pigmented tissues of a wide range of animals. New discoveries of genes regulating these colorful pigments and structures lie on the horizon, with even greater implications for understanding patterns of biodiversity because in many cases, differences in these colors are more clearly involved in delimiting species boundaries and are associated communication signals (section 2.10). We suggest that explorations across a fully integrated spectrum of genes related both to the patterns and colors of pigments and to the perception of these phenotypes within an

ecological and evolutionary context will lead to a deeper understanding of the processes responsible for the evolution of the spectacular diversity of animal coloration we see in nature.

Chapter 3

The relative influence of genes and environment on plumage development demonstrates lifetime effects of early nest environment on a colorful sexual signal²

3.1 Abstract

Phenotypic differences among individuals are often linked to differential survival and mating success. Quantifying the relative influence of genetic and environmental influences on phenotype allows evolutionary biologists to make predictions about the potential for a given trait to respond to selection and various aspects of environmental variation. In particular, the environment individuals experience during early development can have lasting effects on phenotype later in life, such that an individual's phenotype is the product of its genetics and the environments individuals experience during early development, as well as throughout their lives. Here, we leveraged a natural full-sib/half-sib design as well as within-individual longitudinal analyses to examine genetic and environmental influences on plumage color. We find that variation in melanin-based plumage color – a trait known to influence mating success in adult North American barn swallows (*Hirundo rustica erythrogaster*) – is influenced by both genetics and aspects of the developmental environment, including variation due to the maternal phenotype and the nest environment itself. Within individuals, nestling color is predictive of adult color. Accordingly, these early environmental influences are relevant to the sexually-selected plumage color variation in adults. That early environmental conditions have important lifelong implications for individual reproductive performance through sexual signal development lends insight into the information feather color variation conveys to potential mates and competitors in North American barn swallows.

² This work was conducted in collaboration with B.R. Jenkins and R.J. Safran. This manuscript is in review at Heredity.

3.2 Introduction

Morphological signals including horns, antlers, and plumage ornaments are important aspects of an individual's phenotype used to attract mates and defend territories and resources necessary for reproduction (Andersson 1994). Individuals, typically males, use these sexual signals in displays or combat and these signals are consequently linked to reproductive success. An individual's phenotype is the product of both its genotype and the environment in which that genotype is expressed (e.g., Roulin & Dijkstra 2003; Garant et al. 2004; Ingleby et al. 2010; Bolund et al. 2011). Thus, how these traits are shaped by selection and the environmental context in which they are developed and expressed is important for understanding the information content of these traits in addition to how variation in these aspects of phenotype are transmitted from parents to their offspring. Particularly in the case of signal traits that are newly developed each year (e.g., plumage in birds, antlers in mammals), an individual's morphology is not static across developmental stages. While we know very little about the cascading influences of the environment on future signal development, there is evidence that sensitivity to the natal environment has important consequences for an individual's future reproductive success and survival (Merilä & Svensson 1997; Verhulst et al. 1997; Nowicki et al. 2002; Tilgar et al. 2009).

In many oviparous species where development occurs in a discrete nest location, a key aspect of the developmental environment is parental care, which can vary in terms of both quantity and quality within a species. Additionally, the nest environment itself can vary widely in terms of microclimate, the number of siblings in a nest, nest parasites, and many other factors that may impact the development and the expression of traits later in life (see Lindström 1999). For example, in great tits (*Parus major*), nestlings with greater mass are more likely to acquire high quality breeding habitat as an adult (Verhulst et al. 1997). Traits affected by developmental

conditions are not limited to those related to life history and survival; in many insects, conditions during development covary with the expression of secondary sexual ornaments, thus impacting reproductive success (Emlen 1994; Moczek & Emlen 1999; Bonduriansky 2007; Punzalan et al. 2008). Moreover, sexual signals in birds are also known to reflect early environmental conditions. Nutritional status during early development has been shown to influence brain development in birds, which in turn affects song production used in attracting mates and defending territories (Nowicki et al. 1998, 2002). Poor brain development early in life often leads to poor quality song production as an adult, lowering a males ability to acquire a mate (Buchanan et al. 2003; Spencer et al. 2003; MacDonald et al. 2006). The nest environment has also been shown to affect nestling plumage coloration, a trait that is often the target of sexual selection in adult birds. In blue tit nestlings, the development of both structural (UV/blue) and carotenoid-based (yellow) plumage has been shown to be associated with body condition (Johnsen et al. 2003). However, whether these early environment effects on plumage color, in blue tits and other birds, carry over into adulthood – as has been shown for song production – is unknown.

In addition to post-natal developmental conditions, there is extensive evidence that pre-natal environmental conditions (non-genetic maternal effects) significantly impact an individual's phenotype. For example, recent medical research suggests that, in humans, maternal diet during pregnancy can influence an offspring's predisposition to diabetes and obesity during childhood and adolescence as well as their predisposition to associated diseases in adulthood (Rooney & Ozanne 2011). Non-genetic maternal effects (hereafter 'maternal effects') can include elements of the developmental environment, as a female will often select or build a nest in a particular area, brood for particular periods of time, and feed at a particular rate (Räsänen &

Kruuk 2007). However, these maternal effects also extend into the prenatal period dictated by the mother's phenotype, condition, hormone levels, and behavior (Mousseau & Fox 1998; Räsänen & Kruuk 2007; Tschirren et al. 2012). Recently, it has been argued that maternal effects and their influence on offspring phenotype can lead to evolutionary change on an ecological timescale (Mousseau & Fox 1998; Räsänen & Kruuk 2007). Consequently, the maternal environment, as dictated by prenatal maternal effects, is an important factor when assessing the influences on phenotypic variation.

A thorough understanding of the mechanisms that underlie signal trait variation involves knowing 1) the relative influence of past environment, current environment, and genetics on variation in phenotype, 2) how a phenotype changes and develops throughout an individual's lifetime, and 3) how genetics and environment might interact in the production of a phenotype (Danchin 2013). With this information, we can make predictions about the information content of a signal and the processes that maintain trait variation in and among populations. In this study, we ask 1) what is the relative influence of genes and the environment – including the pre-laying maternal environment – on the development of phenotypic variation in plumage color, and 2) how does early environment (i.e., nest environment) influence the development of this trait into adulthood. To address these questions, we quantified the contributions of genetic and environmental variance to the development and expression of a highly variable, continuous melanin-based plumage color trait known to be a target of sexual selection in adults (Safran et al. 2005; Safran et al, in review). To explore the relative significance of genetics and the environment on the development of plumage, we leverage naturally occurring variation in extra-pair offspring to compare phenotypes of related individuals raised in the same and in different nests. Further, we compare variation in the color of individuals at different developmental time

points to assess the role of early environment, or developmental plasticity, in the production of plumage color.

3.3 Methods

3.3.1 Study Species and Study Area

Barn swallows (*Hirundo rustica*) have been a model system for sexual selection research for decades (see Møller, 1994; Turner, 2006). Females of the North American subspecies (*H. r. erythrogaster*) do not attend to long tail streamers in males, but rather to darker melanin-based ventral plumage color (Safran & McGraw 2004; McGraw et al. 2004). Coloration is sexually dimorphic and varies within (Safran & McGraw 2004) and between subspecies (Safran & McGraw 2004; Vortman et al. 2011, 2013; Safran et al, in review). Manipulative experiments conducted in two different populations of North American barn swallows have shown that males whose plumage was experimentally darkened maintain higher paternity in their social broods compared to control males, indicating a causal link between color and reproductive success in multiple North American populations (New York: Safran et al. 2005; Colorado: Safran et al, in review).

During the 2008 and 2009 breeding seasons we monitored barn swallows at 24 breeding sites across Boulder, Jefferson, and Weld counties in Colorado that are part of a large, continental population distributed across North America. We attempted to capture all adults at a site using mist nets and targeted night captures. Adults were banded with United States Geological Survey (USGS) aluminum bands and given a unique color combination that consists of randomly chosen plastic color band and/or colored tail spots that allowed for individual identification during behavioral observations. These color combinations do not have an effect on

an individual's reproductive success (spearman's rho, association between number of fledged young and color band: males rho = 0.090, p = 0.303; females rho = -0.113, p = 0.137; tail spot colors: males rho = -0.123, p = 0.157; females rho = -0.061, p = 0.425). We collected morphological measurements (flattened wing length, tail streamer length, and mass), a blood sample from the brachial vein, and a plumage samples from the breast of the bird. As nests were initiated, we identified the social male and female associated with the nest; documented the clutch initiation date, the clutch size, the hatch date, and the brood size. On day 12 of the nestling period, we banded and measured nestlings (wing length and mass) and took blood and plumage samples. Finally, we estimated when and how many nestlings fledged from successful nests. Despite their socially monogamous mating system, high rates of extra-pair paternity (~30%) have been reported in many populations of barn swallows (Saino et al. 1997; Safran et al. 2005; Kleven et al. 2006); thus we determined whether nestlings were within-pair or extra-pair offspring using microsatellite markers (see below).

3.3.2 Plumage color analyses

Following Safran et al (2010a), feather samples were taped to a standard white card background so that they overlap as they do on the body of a bird. The color of each patch was measured using a spectrometer (USB 4000, Ocean Optics), pulsed xenon light (PX-2, Ocean optics) and SpectraSuite software (v2.0.151). The probe was held perpendicular to the feather surface at a distance such that a 2.5 mm diameter was illuminated and measured. Each sample was measured three times and averaged, with each measurement being an average of 20 scans. From the generated spectra, we quantified color in tetrahedral color space (Stoddard & Prum 2008), which provides three metrics that describe hue (theta and phi) and saturation (r). Theta and phi are measures of angular displacement that describe where in the color space a particular

sample lies; and r describes the distance from the achromatic center of the color space (Stoddard & Prum 2008). As r varies depending on where in the color space a sample is located (theta and phi), here we use r achieved (r_A), which is a measure of r relative to the maximum value of r (r_{max}) possible for that sample. We also quantified average brightness of each plumage sample as a measure of how much light, regardless of wavelength, is reflected off the feather surface (Montgomerie 2006) Across the three measurements for each sample, theta, phi, r_A , and brightness are highly repeatable ($r = 0.93 - 0.95$; $r = 0.91 - 0.93$; $r = 0.82 - 0.87$; $r = 0.88 - 0.93$, respectively). All plumage color metrics were quantified using the R package pavo (Maia et al. 2013) and repeatability was calculated using the ICC package (Wolak et al. 2012).

We report results for all color metrics (theta, phi, r_A , and brightness) as variation in these metrics is likely driven by differing genetic and physiological mechanisms (i.e. total amount of pigment vs. proportion of pigment type) (McGraw et al. 2005; McGraw 2006a; Hubbard et al. 2010).

3.3.3 Paternity analyses

DNA samples were extracted from blood taken in the field using Qiagen DNeasy Blood & Tissue Extraction kits (Maryland, USA). Polymerase chain reaction (PCR) was utilized to amplify seven previously developed microsatellite loci – Escu6: (Hanotte et al. 1994); Ltr6: (McDonald & Potts 1994); Pocc6: (Bensch et al. 1997); and Hir11, Hir19, and Hir20: (Tsyusko et al. 2007); and Hru6: (Primmer et al. 1995). Reaction conditions for pooled Escu6, Ltr6, Hir20, and Hir11 primers consisted of 50-100 ng DNA, 0.12 mM of each labeled forward primer, 0.12 mM of each reverse primer, 200 μ M each dNTP, 3.25 mM $MgCl_2$, 1x PCR Buffer, 0.15 units Taq polymerase (New England Biolabs, Massachusetts, U.S.A.), and were amplified with the following protocol: initial denaturation step of 94°C for 1 minute, followed by 10 cycles of 94°C

for 30 s, 55°C for 30 s, and 72°C for 45 s, with an additional 25 cycles starting at 87°C for 30 s instead of 94°C, and completed with a final extension at 72 °C for 3 min. The Pocc6 reaction was modified from the above conditions by using 1.25 mM MgCl₂, and the above conditions were altered for the Hir19 reaction with 3 mM MgCl₂, and 0.2 mM each forward and reverse primer. The PCR amplification protocol for Pocc6, Hru6 and Hir19 were similar to the previously described protocol for the pooled reaction with the exception that 60°C was used for the annealing temperature. Amplified PCR products containing the fluorescently-labeled forward primer were detected using an ABI3730 DNA analyzer (ABI, Inc.).

Genotypes for nestlings and adults were assigned using GeneMapper software (v4.0, Applied Biosystems). Genotypes from adults and offspring were incorporated into a paternity analysis using CERVUS software (v2.0) to calculate exclusion probabilities and assign nestlings to their social father or to other males in the population. Paternity exclusion was conducted using similar parameters described in Neuman et al. (2007). Briefly, we considered young as extra-pair if we detected two or more alleles (from the seven microsatellite loci) that did not match the social father.

3.3.4 What is the relative influence of genes and environment on juvenile color?

To examine the quantitative genetics of plumage color, we used the animal model, a mixed-effects model that partitions phenotypic variance into different components, such as environmental, genetic, and maternal effects, from which heritability and other parameters can be estimated (Lynch & Walsh 1998; Kruuk 2004; Wilson et al. 2010). This analytical tool has traditionally been used in animal husbandry where pedigrees are closely monitored. With the increasing ease of molecular paternity analyses, it has become a popular tool for natural systems. We estimated the variance components for each color metric by fitting a multivariate animal

model using a Bayesian Markov Chain Monte Carlo (MCMC) technique implemented in the R package MCMCglmm (Hadfield 2010). To estimate relatedness among individuals in order to partition genetic variance, the animal model includes a pedigree term. Our pedigree consisted of 512 offspring with 108 mothers and 95 fathers with one individual represented as both offspring and mother for a total of 715 identities total. We do not have any information regarding the relatedness of the mothers and fathers in this pedigree; thus, we assumed that breeding adults are unrelated. We feel confident in this assumption as individuals recruited into the population are rarely related (Jenkins et al. 2014).

In the model, we initially included year, sampling date, nestling mass, and nestling sex as fixed effects; however, none of these variables had a statistical effect on the model, therefore we did not include any fixed effects in our final model (Wilson *et al.*, 2010; table 3.1). To partition total phenotypic variance into additive genetic variance and nest environmental variance we included the following random effects 1) pedigree and 2) nest identity. Total phenotypic variance (V_P) was calculated as the sum of the variance components: $V_P = V_A + V_{CE} + V_R$. Where V_A is

Table 3.1. Posterior mode (and 95% BCI) of all fixed effects included in maximal model (DIC: All Families = -3216.179; Multiple Broods = -829.254). None of the fixed effects had a statistical effect (posterior distributions overlap zero) and were therefore not included in the final models.

	All Families (95% BCI)	Females with Multiple Broods (95% BCI)
Year	-0.002 (-0.030 – 0.029)	-0.005 (-0.087 – 0.071)
Sampling Date	-0.0001 (-0.0006 – 0.0006)	9.42×10^{-5} (-0.002 – 0.002)
Sex	-0.007 (-0.017 – 0.008)	-0.005 (-0.033 – 0.023)
Body Mass	0.0009 (-0.004 – 0.004)	-0.001 (-0.008 – 0.010)

the additive genetic variance, V_{CE} is the nest environmental variance, and V_R is the residual variance (Falconer & Mackay 1996; Lynch & Walsh 1998). Using the variance components, we calculated narrow sense heritability ($h^2 = V_A/V_P$), and the effect of nest environment ($ce^2 = V_{CE}/V_P$). An advantage of the Bayesian framework used here is that the uncertainty associated with each component carries over into the subsequent variance ratio estimates allowing for Bayesian credible intervals (BCI) to be estimated for heritability and nest environmental effects for each color metric. As there are significant phenotypic correlations among color metrics within individual nestlings (table 3.2), we also estimated the genetic correlation among all pairwise combinations of the four color metrics of the breast plumage.

Table 3.2. Comparison of within-individual phenotypic correlations (below diagonal) and genetic correlations (above the diagonal) among all pairwise combinations of the four color metrics. Significant correlations are in bold. No significant genetic correlations were found with the dataset limited to females with multiple broods used to estimate maternal effects

	Theta	Phi	r_A	Brightness
Theta	---	0.012 (-0.120 - 0.122)	-0.032 (-0.159 - 0.081)	-0.055 (-0.159 - 0.074)
Phi	-0.275 (-0.353 - -0.193)	---	0.035 (-0.095 - 0.148)	-0.051 (-0.184 - 0.058)
r_A	-0.475 (-0.539 - -0.405)	0.238 (0.155 - 0.319)	---	-0.128 (-0.247 - -0.001)
Brightness	0.422 (0.348 - 0.491)	-0.496 (-0.559 - -0.428)	-0.744 (-0.780 - -0.703)	---

We also used a subset of our data for which females had multiple broods within or across breeding seasons allowing us to assess the effect of pre-laying maternal environment separate from nest environment. This pedigree consisted of 246 offspring, 32 mothers, and 45 fathers for a total of 323 identities. As with the complete dataset, we first included year, sampling date,

nestling mass, and nestling sex as fixed effects, however these did not have a statistical effect on the model and were therefore not included in the final model (table 3.1). In addition to pedigree and nest identity, we included maternal identity as random effects. The effect of pre-laying maternal environment was calculated in the same manner as heritability and the effect of nest environment ($me^2 = V_{ME}/V_P$), where $V_P = V_A + V_{CE} + V_{ME} + V_R$.

We specified the priors for variance-covariance matrix as an inverse Wishart matrix distribution (de Villemereuil 2012). For both sets of analyses, we varied the priors specified in the final models by adjusting the inverse Gamma and Beta distributions for variances and correlation; the model outcomes were relatively insensitive to prior parameterization (table 3.3). All models were run for 502,000 iterations, with a burn-in of 2,000 iterations, and every 200th iteration was stored (autocorrelations were weaker than 0.063 for all variance components) with effective-samples sizes between 2307 and 2896.

3.3.5 Does juvenile coloration predict adult signal variation?

As in many migratory passerines, recruitment of juvenile barn swallows into their natal population is low (approximately 1% in our study population) and comparisons of adult color between individuals of known relatedness are therefore extremely difficult. However, nestlings begin to grow juvenile feathers with qualitatively similar coloration to that of adults while they are still in the nest. Since we began monitoring this population in 2008, a small number of individuals banded and sampled as nestlings have returned as breeding adults in their first year (total for which we have plumage samples at both time points through 2012: $n = 54$; males = 41 and females = 13). Using these individuals, we modeled the linear relationship between color measured within the same individual at two different stages: as a nestling and as an adult in their first breeding season to determine if juvenile plumage color predicted adult color within

individuals. Barn swallows go through their first basic molt on the wintering grounds before their first breeding season; consequently, the plumage samples taken from adults in their first breeding season were grown at a different time and place than plumage samples taken from juvenile birds; thus, any within-individual similarity in coloration cannot be due to common environment. All statistical analyses were performed in R v3.0.3 (R Team, 2012).

Table 3.3. Summary of model outcomes using various priors; model outcomes were relatively insensitive to the prior parameterization.

Prior Parameterization:	Final	Gamma = 0.5	Gamma = 0.1	Gamma = 0.01	
DIC:	-3221.14	-4333.413	-6204.549	-7113.941	
Theta	h^2	0.279	0.276	0.294	0.329
	(95% BCI)	(0.232 - 0.32)	(0.237 - 0.33)	(0.25 - 0.356)	(0.254 - 0.461)
	e^2	0.481	0.494	0.443	0.32
	(95% BCI)	(0.427 - 0.557)	(0.419 - 0.549)	(0.381 - 0.519)	(0.244 - 0.415)
Phi	h^2	0.284	0.287	0.308	0.332
	(95% BCI)	(0.242 - 0.333)	(0.243 - 0.34)	(0.254 - 0.378)	(0.209 - 0.478)
	e^2	0.475	0.461	0.401	0.265
	(95% BCI)	(0.414 - 0.546)	(0.401 - 0.536)	(0.334 - 0.479)	(0.2 - 0.369)
r_A	h^2	0.291	0.283	0.309	0.301
	(95% BCI)	(0.236 - 0.33)	(0.24 - 0.336)	(0.25 - 0.377)	(0.188 - 0.472)
	e^2	0.489	0.483	0.374	0.269
	(95% BCI)	(0.415 - 0.546)	(0.405 - 0.535)	(0.327 - 0.474)	(0.169 - 0.353)
Brightness	h^2	0.282	0.295	0.31	0.302
	(95% BCI)	(0.243 - 0.336)	(0.244 - 0.347)	(0.249 - 0.398)	(0.16 - 0.493)
	e^2	0.481	0.459	0.369	0.256
	(95% BCI)	(0.408 - 0.54)	(0.385 - 0.528)	(0.303 - 0.46)	(0.167 - 0.368)

3.4 Results

3.4.1 Relative influence of genes and environment during juvenile plumage development

Leveraging mixed paternity within the nest to analyze the influence of genes and the environment on trait development. We assigned genotypes for 512 nestlings and 125 parental pairs for all seven loci. With a combined first-parent exclusion probability of 99.88% for all seven loci, we were able to assign 303 nestlings as within-pair young (sired by social father), and 209 as extra-

pair young (not sired by social father). Of those 209 extra-pair young, we were able to determine the identity of the extra-pair father for 63 nestlings. Based on all families genotyped in the 2008 and 2009 breeding seasons, we found a high rate of extra pair paternity in our study population, with, on average, 40% of nestlings sired by non-social males during the 2008 (44.3%) and 2009 (36.6%) breeding seasons. In both 2008 and 2009, approximately 65% of nests contained at least one extra-pair young (EPY), with some nests having 100% EPY (fig. 3.1). These percentages are consistent with what is found in the dataset used here.

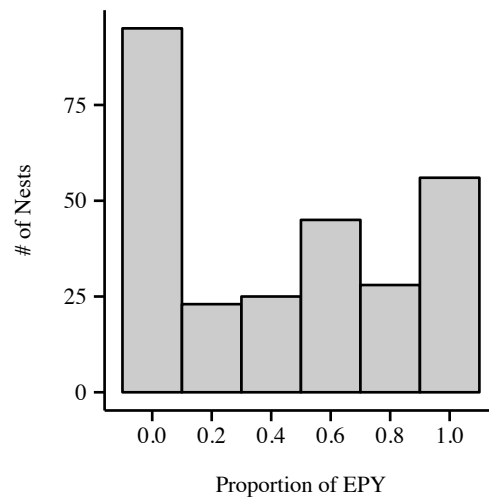


Figure 3.1. Histogram showing the proportion of extra-pair young (EPY) in a nest; data pooled across 2008 and 2009 breeding seasons.

The model included pedigree and nest identity as random effects to partition phenotypic variance into additive genetic variance and nest environmental variance (table 3.4). From these variance components, we calculated variance ratios to determine the relative effect of shared genes (h^2) and shared nest environment (ce^2) for each color descriptor (table 3.5).

Shared Genes. The high occurrence of EPY in our populations created a half-sib/full-sib structure among offspring. In this population, relatedness explains approximately 28% of the phenotypic variation in melanin-based plumage coloration (table 3.5). Additionally, despite significant within-individual phenotypic correlations among the four color metrics, we did not find evidence of strong genetic correlations for these four traits. Average brightness and r_A were significantly genetically correlated but the correlation coefficient was extremely small suggesting a weak effect (table 3.2).

Table 3.4. Posterior modes of variance components (and 95% BCI) for each color metric from a multivariate animal model.

		All Families			
Variance Component		Theta (95% BCI)	Phi (95% BCI)	r_A (95% BCI)	Brightness (95% BCI)
V_A	Additive genetic	0.012 (0.01 - 0.014)	0.012 (0.01 - 0.015)	0.013 (0.011 - 0.015)	0.013 (0.011 - 0.016)
V_E	Nest environment	0.02 (0.016 - 0.026)	0.022 (0.016 - 0.027)	0.021 (0.016 - 0.027)	0.022 (0.018 - 0.029)
V_R	Residual	0.009 (0.008 - 0.011)	0.01 (0.009 - 0.012)	0.01 (0.009 - 0.012)	0.011 (0.01 - 0.013)
V_P	Total Phenotypic	0.042 (0.036 - 0.049)	0.044 (0.038 - 0.051)	0.044 (0.038 - 0.051)	0.047 (0.042 - 0.055)

Shared Nest Environment. While most related individuals also shared the same nest environment, our dataset consisted of several maternal and paternal half siblings that experienced different environments. We determined that nest environment explains a larger proportion (approximately 48%), relative to relatedness (approximately 28%), of phenotypic variation in coloration (table 3.5).

Table 3.5. Posterior modes of variance ratio estimates (and 95% BCI) for each color metric estimated from a multivariate animal model (DIC = -3216.179). Variance ratios were calculated as follows: narrow sense heritability ($h^2 = V_A/V_P$) and nest environment ($ce^2 = V_{CE}/V_P$). Theta and phi are measures of hue, r_A is a measure of saturation, and brightness is a measure of reflected light.

Ratio	Definition	Theta (95% BCI)	Phi (95% BCI)	r_A (95% BCI)	Brightness (95% BCI)
h^2	V_A/V_P Proportion of total phenotypic variance explained by additive genetic variance	0.279 (0.232 - 0.320)	0.284 (0.242 - 0.333)	0.291 (0.236 - 0.330)	0.281 (0.243 - 0.336)
ce^2	V_{CE}/V_P Proportion of total phenotypic variance explained by nest environment	0.481 (0.427 - 0.557)	0.475 (0.414 - 0.546)	0.489 (0.415 - 0.546)	0.481 (0.408 - 0.540)

Maternal Effects. Using a subset of the data for which mothers had multiple broods, the model included pedigree, nest identity, and maternal identity as random effects to partition phenotypic variance into additive genetic variance, nest environmental variance, and pre-laying maternal environmental variance (table 3). From these variance components, we calculated variance ratios to determine the relative effects of shared genes (h^2), shared nest environment (ce^2), and shared maternal environment (me^2) for each color descriptor (table 3.6).

Table 3.6. Posterior modes of variance ratio estimates (and 95% BCI) for each color metric estimated from a multivariate animal model (DIC = -829.254). Variance ratios were calculated as follows: narrow sense heritability ($h^2 = V_A/V_P$), nest environment ($ce^2 = V_{CE}/V_P$), and pre-laying maternal environment ($me^2 = V_{ME}/V_P$).

Ratio	Definition	Theta (95% BCI)	Phi (95% BCI)	r_A (95% BCI)	Brightness (95% BCI)
h^2	V_A/V_P Proportion of total phenotypic variance explained by additive genetic variance	0.126 (0.085 – 0.179)	0.134 (0.089 – 0.184)	0.128 (0.086 - 180)	0.144 (0.090 - 186)
ce^2	V_{CE}/V_P Proportion of total phenotypic variance explained by nest environment	0.276 (0.172 – 0.375)	0.262 (0.175 – 0.370)	0.278 (0.181 – 0.379)	0.249 (0.175 – 0.368)
me^2	V_{ME}/V_P Proportion of total phenotypic variance explained by maternal environment	0.456 (0.361 – 0.646)	0.474 (0.351 – 0.625)	0.480 (0.349 – 0.628)	0.482 (0.355 – 0.631)

When maternal identity is included in the model, the phenotypic variation explained by both relatedness and nest environment decreases (approximately 13% and 26%, respectively), and the majority of phenotypic variation in coloration is explained by pre-laying maternal environment (approximately 46%, table 3.4).

3.4.2 Juvenile coloration predicts adult signal

Differences between nestlings and adults. We explored age and sex differences in plumage color using two-way ANOVAs for each color metric (theta, phi, r_A , and average brightness). In each model, the interaction term was significant (theta: $F_{1, 1914} = 59.06$, $p < 0.0001$; phi: $F_{1, 1914} = 77.03$, $p < 0.0001$; r_A : $F_{1, 1914} = 11.86$, $p < 0.001$; and brightness: $F_{1, 1914} = 19.36$, $p < 0.0001$), and we used a Tukey's post hoc analysis to assess the pairwise comparisons of interest. In both nestlings and adults, we found sexual dichromatism illustrated by significant differences between the sexes in all four color metrics; on average, males are darker with more saturated color (higher r_A). Additionally, differences among males and females for hue (theta and phi) are in the same direction suggesting variation within color space between the sexes is similar at both developmental stages (table 3.5; fig. 3.2). In females, adults and nestlings significantly differ in all four color metrics, however in males, significant differences were only found in theta and r_A , with phi and brightness not differing between the developmental stages (table 3.7; fig. 3.2).

While these differences are statistically significant, figure 3.1 illustrates that some differences are

Table 3.7. Pairwise differences (and 95% CI) from Tukey's post hoc analysis comparing color among sex and developmental stages. Significant differences are in bold.

	Theta	Phi	r_A	Brightness
	Males vs. Females	Males vs. Females	Males vs. Females	Males vs. Females
Nestlings	-0.014 (-0.022 - -0.005)	0.016 (0.003 - 0.029)	0.017 (0.004 - 0.031)	-2.988 (-4.385 - -1.591)
Adults	-0.046 (-0.053 - -0.061)	0.073 (0.063 - 0.087)	0.041 (0.03 - 0.056)	-6.082 (-7.229 - -9.319)
	Nestlings vs. Adults	Nestlings vs. Adults	Nestlings vs. Adults	Nestlings vs. Adults
Males	-0.01 (-0.018 - -0.003)	-0.008 (-0.02 - 0.003)	-0.181 (-0.194 - -0.169)	1.07 (-0.203 - 2.349)
Females	-0.043 (-0.051 - -0.053)	0.049 (0.037 - 0.04)	-0.157 (-0.17 - -0.339)	-2.02 (-3.01 - -0.942)

quite small and it is unclear whether they are biologically relevant differences or an artifact of relatively large sample sizes.

Nonrandom recruitment of nestlings as breeders? For the color comparisons between recruited and non-recruited individuals, we used Welch's two-sample t-test, which assumes unequal variance in groups. This analysis is appropriate as our sample sizes for the two groups were unequal (recruited = 54; non-recruited nestlings = 2625; non-recruited adults = 1329). We found no statistically significant difference in plumage coloration between nestlings that were recruited into their natal population compared to entire nestling population from the same years (theta: $t_{57.26} = -0.046$, $p = 0.963$ fig 3.3A; phi: $t_{55.46} = -0.292$, $p = 0.771$ fig. 3.3D; r_A : $t_{54.72} = 1.94$, $p = 0.058$ fig. 3.3G; brightness: $t_{54.68} = -1.51$, $p = 0.138$; fig. 3.3J); therefore, we infer that the recruited nestlings are a random subset of the nestlings hatched in our population. As adults, individuals hatched in our study area and recruited into the breeding population generally did not differ in plumage color compared to all first time breeding adults (theta: $t_{59.74} = -1.537$, $p = 0.130$ fig 3.3B; r_A : $t_{55.39} = -0.827$, $p = 0.412$ fig. 3.3F; brightness: $t_{57.16} = -0.389$, $p = 0.699$; fig. 3.3K), however there was a significant difference in one of the metrics of hue, phi ($t_{60.44} = 2.866$, $p = 0.006$ fig. 3.3E).

Longitudinal analyses: predicting color across years and life stages. Within an individual, 12-day old nestling plumage color significantly predicted adult plumage color in their first breeding season. We found significant relationships in all four color metrics with the measures of chroma and brightness showing the strongest relationships, and the two measures of hue being less predictive ($r_A - b = 0.848$, $R^2 = 0.264$, $F_{1, 52} = 20.01$ $p < 0.0001$, fig. 3.3I; average brightness - $b = 0.380$, $R^2 = 0.177$, $F_{1, 52} = 12.41$, $p < 0.001$, fig. 3.3L; theta - $b = 0.278$, $R^2 = 0.022$, $F_{1, 52} = 2.184$, $p = 0.146$, fig. 3.3C; phi - $b = 0.252$, $R^2 = 0.024$, $F_{1, 52} = 2.306$, $p = 0.135$, fig. 3.3F). The

finding that nestling color predicts adult color indicates that early environment has lasting effects on future plumage color development in barn swallows.

3.5 Discussion

In barn swallows, variation in plumage color is predictive of individual reproductive performance (Safran & McGraw 2004; Safran et al. 2005; Safran et al, in review). Here, we found that variation in juvenile plumage color is primarily affected by nest environment and maternal effects and that juvenile plumage color predicts an individual's plumage color as a first-time breeding adult. Taken together, these results indicate that the environment an individual experiences as a developing nestling has long-term effects on sexual signal development and therefore, on reproductive performance. In this scenario, we would predict that darker males would sire darker sons, however, an experiment that decouples nest quality from male color is needed to differentiate between these two hypotheses.

3.5.2 Heritability of Melanin-Based Color

In contrast to other studies that estimate heritability of melanin-based plumage color (Grant 1990; Mundy 2006; Potti & Montalvo 2008; Saino et al. 2013), our study reveals fairly low heritability for plumage color in North American barn swallows ($h^2 \cong 0.28$). In a different population of barn swallows, Saino *et al* (2013) found that ventral plumage color was highly heritable ($h^2 \cong 0.80$) using parent-offspring regressions. However, analyses like parent-offspring regression that do not control for other sources of non-independence such as shared environment, may overestimate heritability. Consequently, the animal model approach used here allows for a more accurate partitioning of phenotypic variance (Kruuk 2004; Wilson et al. 2010). Moreover, in this study, we exploited a natural half-sib/full-sib structure created by a high rate of extra-pair

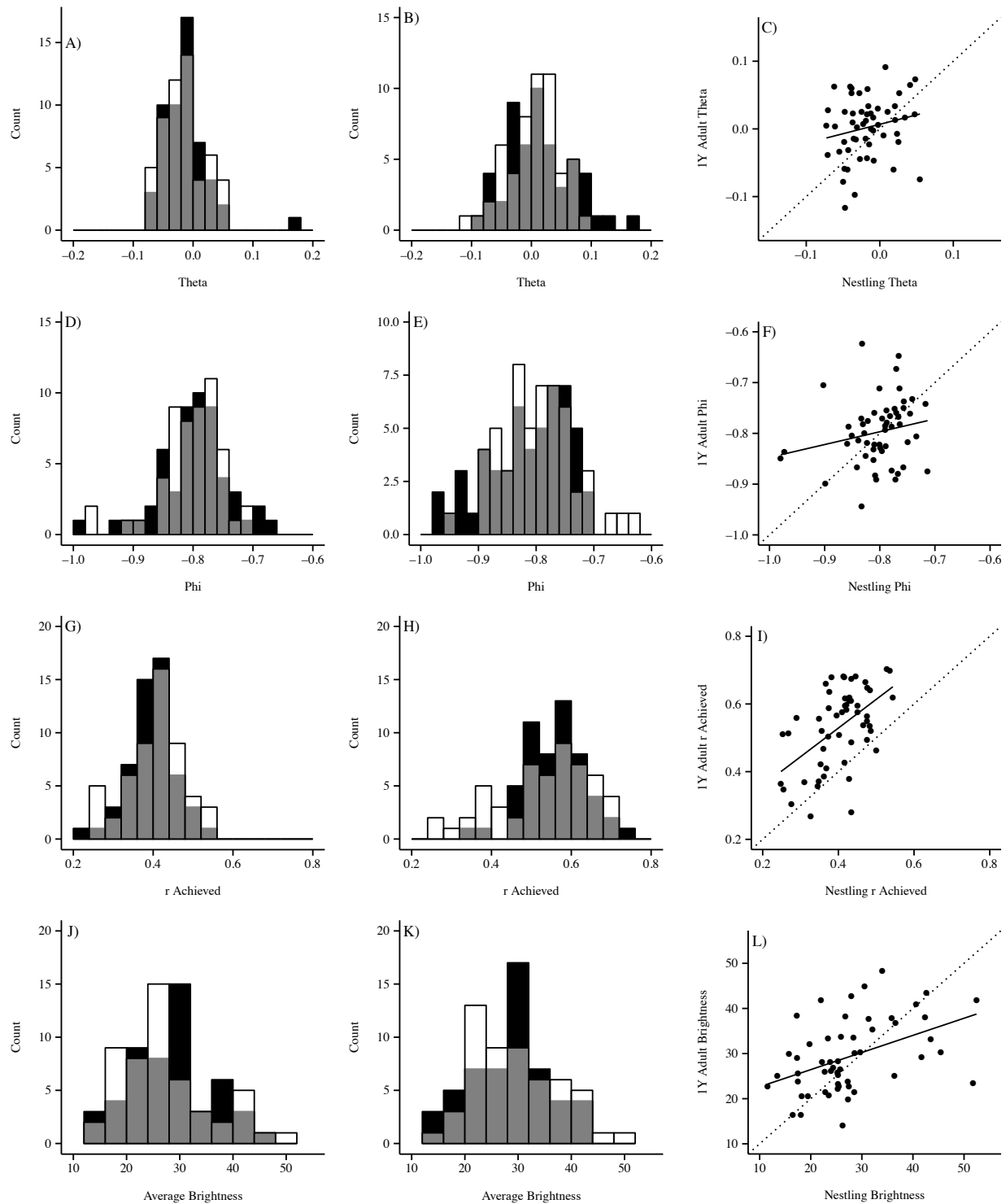


Figure 3.3. Individuals recruited into their natal population are a random subset of the population and within-individuals color is predictive from one developmental stage to the next. Panels A, D, G, and J show that the distribution of each nestling color metric for the 54 returning individuals (white bars) is no different from a random selection of 54 nestlings from the entire population (black bars). Panels B, E, H, and K show that the distribution of each adult color metric for the 54 returning individuals (white bars) is no different from a random selection of 54 adults from the entire population (black bars). The gray bars indicate overlap in the distributions. When compared to the entire adult population (rather than a random subset), phi does significantly differ. In panels C, F, I, and L we are showing that nestling color is predictive of adult color within an individual.

young (~40%) that allowed us to estimate the effects of genetic and environmental variation analytically. However, in our study system, related individuals are likely to experience the same nest and pre-laying maternal environments; consequently, our estimates for heritability and developmental environmental effects may be confounded such that phenotypic variation due to additive genetic variation is being attributed to environmental variation, yielding low heritability estimates. Future work to experimentally isolate genetic and environmental effects (i.e., cross-fostering experiments) will be quite illuminating (Lindström 1999).

3.5.3 Developmental Plasticity

Our study also demonstrates that within an individual, plumage color during development is highly predictive of plumage color as a first time breeding adult. This is particularly interesting given that plumage color is developed several times within an individual's lifetime: first, in the natal environment on breeding grounds in North America and subsequently, once per year during the nonbreeding season in Central and South America before they migrate back to breeding sites. If an individual's underlying genotype explained this pattern of within-individual variation, we would expect much higher heritability estimates with related individuals having highly similar phenotypes as they are more likely to have the same underlying genotype. Therefore, we infer there is developmental plasticity for melanin-based plumage color as a function of the nest environment, and this plasticity has long-term effects such that nest environment influences the adult phenotype despite a subsequent molt after leaving the nest environment.

In birds, developmental conditions have been shown to affect many aspects of an individual's phenotype and fitness, including survival (Merilä & Svensson 1997), future clutch size (Haywood & Perrins 1992), and the ability to obtain and defend high quality breeding habitat (Verhulst et al. 1997). Additionally, early conditions can have significant impacts on

important sexual signals such as song (Nowicki et al. 1998), plumage traits (Scordato et al. 2012), and morphology (Ohlsson et al. 2002). Here, we show that the environmental conditions experienced by a nestling barn swallow during the first few weeks of life affect the development of a colorful sexual signal known to affect reproductive success (Safran *et al.*, 2005; Safran et al, in review).

3.5.4 Maternal Effects

This study additionally revealed that variation in plumage color was largely explained by the pre-laying maternal environment, which differs from the nest environment as related offspring raised in different nesting attempts within and across breeding seasons will experience different nest conditions, but are likely to experience the same pre-laying maternal environment. For example, a female's condition and phenotype can influence hormone deposition in eggs, which is known to vary among female barn swallows (Safran et al. 2008b), as well as other passerines (Groothuis & von Engelhardt 2005; Müller et al. 2012). However, in this study, it is impossible to differentiate between non-genetic prenatal effects such as hormone deposition and postnatal behavioral effects such as parental care. Consequently, the decrease in variation explained by nest environment when pre-laying maternal environment is included in the model may be the result of similar parental care behaviors. An experiment where individuals experience the same pre-laying maternal environment, but are raised by unrelated females (or parent pairs) would help clarify these relationships (e.g., White *et al.*, 1968; Beamonte-Barrientos *et al.*, 2010).

3.5.5 Early Environment Impacts a Sexually Selected Trait

A causal relationship between color and paternity exists in two populations of North American barn swallows, such that darker males are allocated more paternity by their social mate

(Safran *et al.*, 2005; Safran *et al.*, in review). Plumage color is unique in that it is redeveloped annually, and consequently subject to environmental influences during regrowth. Results from this study suggest that rather than signaling how a male is impacted by the current (or recent) environmental context, male color may provide information about conditions, including maternal effects, an individual experienced during early development. Moreover, developmental conditions may impact other behavioral, physiological, and morphological traits that affect female mate choice. For example, empirical support for the Nutritional Stress Hypothesis (Nowicki *et al.* 1998, 2002) shows that early developmental conditions have drastic effects on song learning and production later in life in many species of songbirds. As song is often the target of sexual selection via mate choice, the Nutritional Stress Hypothesis may provide a mechanism for maintaining the reliability of a key sexual signal; a similar mechanism (early developmental conditions) could maintain the reliability of plumage color in barn swallows.

3.6 Conclusions

Ventral plumage coloration in North American barn swallows is representative of many melanin-based color traits with continuous variation. Results from this study demonstrate that both the underlying genes and the environment in which feathers are developed influence juvenile plumage color. Moreover, early environment during development (through maternal effects and features of the nest environment) have lasting effects on adult phenotype, a pattern that has not been previously shown for melanin-based plumage color. Ventral plumage coloration in adult male swallows is known to impact reproductive success in terms of differential paternity allocation by mates (Safran *et al.*, 2005; Safran *et al.*, in review); consequently, early nest environment likely has long-term effects on an individual's lifetime

fitness. Given this link between sexually-selected plumage color and an individual's developmental environment, females may use this trait in mate choice decisions because it can convey information about the early developmental conditions a male has experienced. Moreover, because of the influence of the early environment on signal development, the nest site may be an important feature of mate choice in this system. Future work aimed at more finely disentangling the roles of genetic and environmental variation on the development of this trait via cross-fostering experiments will enable researchers to causally isolate the effects of these two sources of variation and identify specific environmental factors impacting signal development.

Chapter 4

Heritability of melanin-based coloration and its implications for mechanisms of sexual selection³

4.1 Abstract

Female choice models of sexual selection are founded on the idea that females gain some benefit from choosing to mate with a particular male. These benefits can be direct, such as protection from predators or high quality habitat, and in these cases the female herself is the beneficiary. Alternatively, benefits can be indirect, such as locally adapted alleles, and the beneficiaries are the offspring. Traits used by females to select a mate should reflect his ability to provide these benefits. Consequently, traits that signal direct benefits should be linked to condition or the environment while traits that signal indirect benefits should be more closely linked to genetic variation. In this study, I explore the influence of genetic and environmental variation on melanin-based plumage coloration in North American barn swallows (*Hirundo rustica erythrogaster*) with the goal of inferring the mechanism of sexual selection affecting this trait. I found that coloration is strongly influenced by the environmental variation, including the early environment (i.e., incubation) suggesting that coloration is indicative of an individual's developmental conditions. While heritability is low, females may still base mate choice on coloration to gain 'good genes' for their offspring, and this may be especially important when females select extra-pair mates.

³ This work was conducted in collaboration with A.K Hund and R.J. Safran.

4.2 Introduction

Sources of phenotypic variation can broadly be described as genetic or environmental, and the relative contributions of these factors can help predict a population's likely response to selection and variable environmental conditions (Barrett et al. 2008; Linnen et al. 2009; Hubbard et al. 2010; McKinnon & Pierotti 2010). It is particularly useful to consider these sources of phenotypic variation for sexually selected traits. Whereas natural selection is defined by traits which confer differential survival (Darwin 1859), sexual selection is defined by traits which confer various features of differential mating success (Andersson 1994). There are many models of sexual selection, each with different predictions about the relative importance of genes and environment for trait development (Safran et al. 2013).

From the perspective of female choice models of sexual selection, traits used in mate choice can provide a female with indirect benefits, such as good genes for her offspring (Fisher 1958; Kirkpatrick 1996), or direct benefits, such as good paternal care (Iwasa & Pomiankowski 1999; Kokko et al. 2003). A trait that signals indirect benefits should be highly heritable, such that advantageous alleles are passed on to offspring (table 4.1, G alone). Alternatively, a trait that signals direct benefits should be more environmentally mediated relative to one that signals indirect benefits because females are basing mate choice decisions on a male's ability to provide or perform in the current environmental context (table 4.1, E alone). Many traits fall along a continuum between these extremes (G only or E only), as multiple mechanisms of sexual selection can act simultaneously (table 4.1, G + E). Thus, assessing the relative influence of genetic and environmental variation on phenotypic variation can provide insight into the mechanism of and benefits associated with sexual selection acting on a trait used for intersexual mate choice.

In many animal taxa, colorful traits are highly variable and often used in sexual interactions such as mate choice or intrasexual competition (Safran et al. 2005; Amundsen & Pärn 2006; Senar 2006; Protas & Patel 2008). These colors are most often the result of pigment deposition, of which melanins and carotenoids are the most common (Hubbard et al. 2010). Historically, carotenoid based-colors have been thought to be more sensitive to environmental conditions, while melanin-based colors were under strong genetic control. Empirical support for this dichotomy exists (reviewed in Griffith et al. 2006), yet there is mounting evidence for environmental dependence of melanin-based colors (Griffith et al. 2006; McGraw 2006d, 2008; Lindsay et al. 2011). However, little work has been done to explore the factors that create the subtler variation of quantitative traits like continuous color variation within a population on which selection is likely to act.

In this study, I use a powerful cross-fostering experiment to quantify the contributions of genetic and aspects of environmental variance to the development and expression of a highly variable, continuous melanin-based plumage color trait known to be a target of sexual selection (Safran et al. 2005; Safran et al., in review). I also estimate narrow-sense heritability (h^2) and the effect of environment for melanin-based plumage color to test hypotheses about the relative influence or interaction of genetics and the environment (table 4.1).

4.3 Methods

4.3.1 Study Species and Study Area

Barn swallows (*Hirundo rustica*) have been a model system for sexual selection research for decades. Females of the North American subspecies (*H. r. erythrogaster*) do not attend to long tail streamers in males (as seen in European populations (Møller 1994; Turner 2006), but rather

Table 4.1. Identifying the relative influence of genetics and environment on phenotypic variation can indicate how a trait will respond to selection and what mechanism(s) of sexual selection might be acting on that trait. These scenarios represent extremes along a continuum, and the relative influence of genetics and environment can vary anywhere between complete genetic control on phenotype to complete environmental control on phenotype.

Influence G and E on phenotype	Prediction	Phenotypic variation explained by	Response to Selection	Response to Variable Environment	SS Mechanism
G alone	Parental phenotypes are highly predictive of offspring phenotype. Shared genes lead to similar phenotypes	Shared genes (h^2)	Phenotype will change over time in relation to selective pressure	None	Indirect benefits: good genes or sexy son
G + E	Parental phenotypes are predictive of offspring phenotype. Shared genes and/or shared environment lead to similar phenotypes	Shared genes (h^2) and shared environment (ee^2 , re^2)	Phenotype will change over time in relation to selective pressure	Population level variation in phenotype will vary over space and time in relation to environmental variation	Indirect and/or Direct benefits
E alone	Parental phenotypes are not predictive of offspring phenotype. Shared environments lead to similar phenotypes	Shared environment (ee^2 , re^2)	None	Population level variation in phenotype will vary over space and time in relation to environmental variation	Direct benefits: e.g., parental care, low disease transmission risk, territory quality

to darker melanin-based ventral plumage color (Safran & McGraw 2004; McGraw et al. 2004). Coloration is sexually dimorphic and varies within and between subspecies (Safran & McGraw 2004). Manipulative experiments conducted in two different populations of North American barn swallows have shown that artificially darkened males maintain higher paternity in their social broods compared to control males, indicating a causal link between color and reproductive success in multiple North American populations (New York: Safran et al. 2005; Colorado: Safran et al in review). Identifying the relative influence of genes and environment on ventral plumage color will further illuminate whether females are gaining direct benefits, indirect benefits, or both by choosing males as a function of color variation (see table 4.1).

4.3.2 Color measurements

Plumage samples were taken from all nestlings on day 12 of the nestling period. Plumage color was measured with a photospectrometer (see chapter 3 for details) and quantified in tetrahedral color space (Stoddard & Prum 2008) using pavo (Maia et al. 2013). Four color metrics are quantified – theta and phi are angle measurements that dictate where a sample is found in the color space and provide information about a sample's hue, R_A is a measure of saturation, and brightness is an achromatic measure of light reflectance. Within an individual, nestling color is predictive of adult coloration (see chapter 3), therefore, nestling color can serve as a proxy for adult color in manipulative experiments.

4.3.3 Cross-Fostering Experiment

During the 2012 breeding season I monitored barn swallows at 26 sites across Boulder, Jefferson, and Weld counties in Colorado that form a larger breeding population. I attempted to capture all adults at a site using mist nets and targeted night captures. Adults were banded, marked, and measured in the same manner as explained in chapter 3. As nests were initiated, I

identified the social male and female associated with the nest; documented the clutch initiation date, the clutch size, the hatch date, and the brood size.

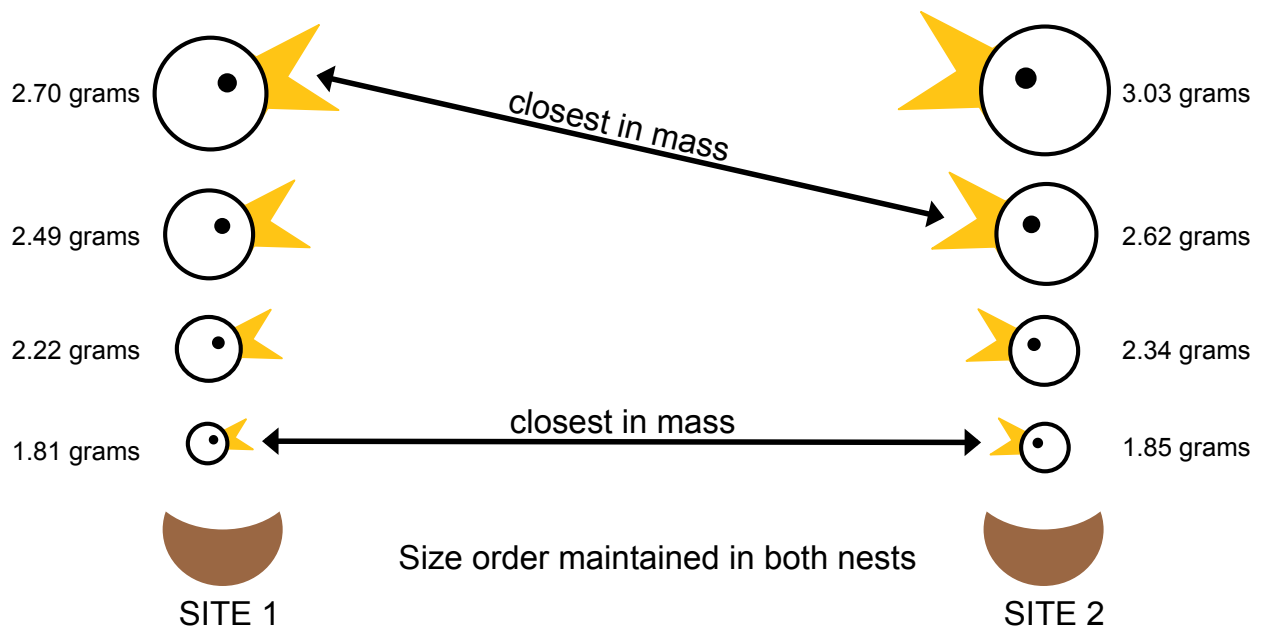


Figure 4.1. Diagram of cross-fostering procedure that took place on day 2 of the nestling period

I paired nests across nesting sites that hatched on the same day, had at least four nestlings, and brood sizes within one chick. On day two of the nestling period (with hatch day being day zero) I exchanged two nestlings between paired nests (fig. 4.1). To identify the nestlings to be exchanged, all nestlings in both nests were weighed, and the largest and smallest nestlings in each nest were identified. I selected the smaller of the largest in each nest and the larger of the smallest in each nest; I then matched these to nestlings in the other nest that were the closest in mass and exchanged them across the nests. By matching individuals based on mass, I maintained the size rank order in each nest (fig. 4.1). I marked the legs of exchanged nestlings with non-toxic permanent marker. I remarked the legs on day 4, and banded nestlings on day 6. I took the mass of each nestling at day 6 and day 9 of the nestling period. On day 12, which is near fledging, I measured nestlings (wing length and mass) and took blood and plumage

samples (breast only) for paternity and color analyses (described in chapter 3). Finally, I estimated when and how many nestlings fledged from successful nests.

This cross-fostering experiment allows me to better isolate genetic and environmental effects on phenotype by decoupling genetic parents from nest environment (Falconer & Mackay 1996; Slagsvold et al. 2002). A cross-fostering design decouples genetic and environmental influences on a trait by putting related individuals in different environments.

4.3.4 What is the relative influence of genes and environment on juvenile color?

As in chapter 3, I used an animal model to estimate key variance components and calculate the proportion of phenotypic variation in color explained by each component. For each model, sex, date, and right wing length (a proxy for feather development) were initially included as fixed effects (table 4.2). For each color metric, I partitioned variance into additive genetic variance, early nest environment (incubation through the first two days of the nestling period), and rearing nest environment variance (where nestling was raised after first two days). To partition variance into these components, I included the following random effects: pedigree, nest of origin and nest of rearing. The total phenotypic variance (V_P) was calculated as the sum of the variance components: $V_P = V_A + V_{EE} + V_{RE} + V_R$. Where V_A is the additive genetic variance, V_{EE} is the early nest environment variance, V_{RE} is the rearing environment variance, and V_R is the residual variance (Falconer & Mackay 1996; Lynch & Walsh 1998). I included the early environment (incubation and first two days post hatching), as there may be important environmental effects experienced in that early nestling period that influence plumage development.

Using the variance components, I calculated narrow sense heritability ($h^2 = V_A/V_P$), the effect of early environment ($ee^2 = V_{EE}/V_P$), and the effect of rearing environment ($re^2 =$

V_{RE}/V_P). All animal models were fit with the MCMCglmm package in R (Hadfield 2010). All other analyses were performed in R v3.0.3 (Team 2014).

4.4 Results

4.4.1 Cross-Fostering

I exchanged nestlings in 90 nests for a total of 45 experimental nest pairs; I also included 51 control nests in which I handled the nestlings as often as the experimental nests. Due to mortality or incomplete data, I had a final sample size of 86 experimental nests with 271 nestlings, and 43 control nests with 160 nestlings for a total of 431 nestlings. I assigned genotypes for all 431 nestlings and their social parents using six of the seven loci described in chapter 2 (Hru6 not used here) with a combined first-parent exclusion probability of 99.1% for all six loci. Of the 431 nestlings (234 male, 197 female), 211 were within-pair young, 189 were extra-pair young (EPY), and I was unable to determine the status of 31 nestlings due to the male not being captured. Of the 189 EPY, I was able to identify the genetic father for 101 nestlings based on a candidate father breeding at the same site, and having one or no mismatches with the EPY.

There was no difference in survival between experimental nestlings, control nestlings, and the non-experimental nestlings demonstrating that the increased handling and marking had no effect on survival ($X^2 = 2.297$, $df = 3$, $p = 0.513$; fig. 4.2); I found no differences in survival among individuals that were and were not marked with the non-toxic marker in control nests ($X^2 = 0.205$, $df = 1$, $p = 0.650$), consequently, I pooled all control nestlings into a single treatment group. For experimental and control nestlings I took mass measurements throughout the nestling period and I found no difference in growth rate between experimental nestlings

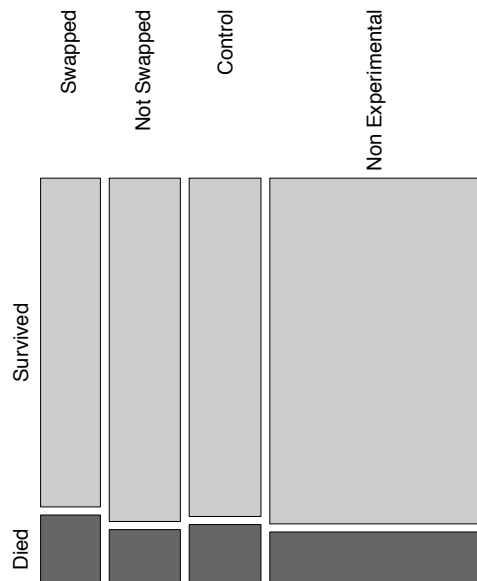


Figure 4.2. Mosaic plot showing no difference in survival among swapped, non-swapped, control, and non-experimental nestlings.

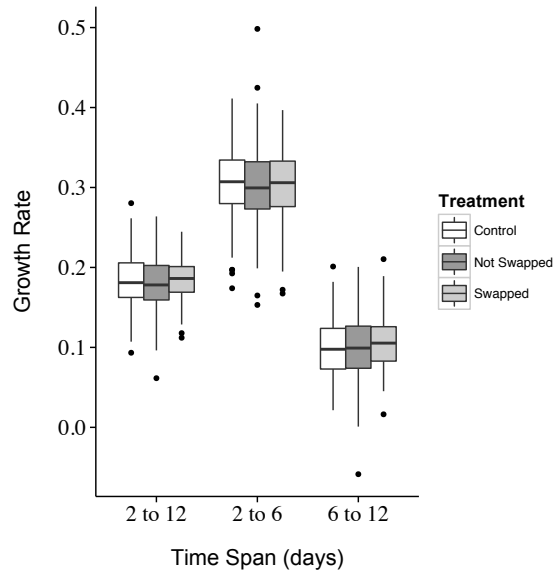


Figure 4.3. Growth rates at 3 different time spans – between days 2 and 12, between days 2 and 6, and between days 6 and 12. There is no difference in growth rate at any time span among the three treatment groups.

(swapped and not swapped) and control nestlings (two-way ANOVA: $F_{2, 1372} = 2.383$, $p = 0.093$; fig. 4.3), although growth rate significantly changed throughout the nestling period for all treatments (two-way ANOVA: $F_{2, 1372} = 2686$, $p < 0.001$; fig. 4.3). Nestling coloration also did not vary with treatment group (Theta: $F_{2, 427} = 0.959$, $p = 0.385$; Phi: $F_{2, 427} = 2.017$, $p = 0.134$; R_A : $F_{2, 427} = 3.037$, $p = 0.049$ – but no significant pairwise differences; $F_{2, 427} = 0.077$, $p = 0.926$; fig. 4.4). These results demonstrate that my experimental procedures did not have effects on survival and growth, and more importantly, that color variation was not influenced by the application of the treatment.

4.4.2 Relative influence of genes and environment on juvenile color

Multivariate Animal Models. The model initially included sex (as both nestlings and adults are sexually dichromatic), sampling date, and right wing length (as a proxy for feather development) as fixed effects, however, none of these had a statistical effect on the model, therefore, I did not

include these terms in the final model (table 4.2). I included pedigree, nest of origin, and nest of rearing as random effects to partition phenotypic variance into additive genetic variance, early environmental variance, and rearing environmental variance (table 4.3). From these variance components, I

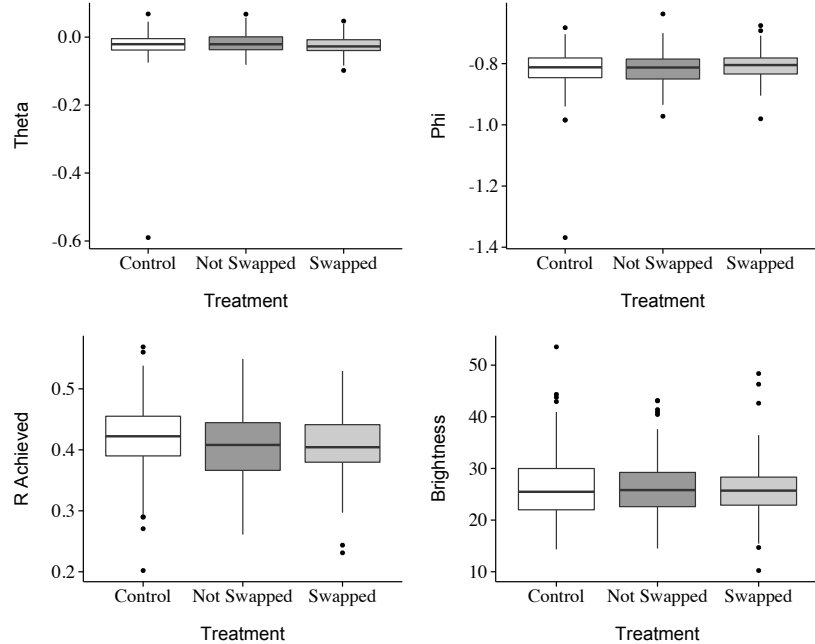


Figure 4.4. Plumage color does not vary in association with treatment.

calculated variance ratios to determine the relative effect of shared genes (h^2), the effect of early environment (ee^2), rearing environment (re^2) for each color descriptor (table 4.2).

Shared Genes. With the cross-fostering design, and the high occurrence of EPY in this population, I created a half-sib/full-sib structure among offspring in which related offspring experienced different rearing environments.

Similar to what was reported in chapter 3 when maternal effects were included in the model, relatedness explains approximately 16% of the phenotypic variation in

Table 4.2. Posterior mode (and 95% BCI) of all fixed effects included in maximal model (DIC: -2156.59). None of the fixed effects had a statistical effect (posterior distributions overlap zero) and were therefore not included in the final models.

	Posterior Mode (95% BCI)
	0.0001
Sampling Date	(-0.001 – 0.001)
	-0.0005
Sex	(-0.017 – 0.018)
	0.0003
Right Wing Length	(-0.002 – 0.002)

melanin-based plumage coloration (table 4.3). I found no significant genetic correlations among pairwise comparisons of color traits.

Shared Environment. Overall, nest environment explains approximately 67% of the phenotypic variation in juvenile color. This can be broken down into two sources of environmental variation: 1) the early environment that nestlings experienced during their first two days post hatching, and 2) the rearing environment they experienced during the remainder of the nestling period.

Early environment. In the early environment, nestlings are cared for (incubation, brooding, and feeding in the first two days) by their original social parents with their siblings. This environment explained approximately 35% of color variation (table 4.3).

Rearing environment. In the rearing environment, a subset of nestlings continue to be cared for by their social parents. However, the nestlings that were exchanged between nests experienced a different nest environment with unrelated parents and nestlings. This environment explained approximately 32% of color variation (table 4.3).

4.5 Discussion

In North American barn swallows, experimentally darkened males are allocated more paternity by their social mate relative to their non-manipulated counterparts (Safran et al. 2005; Safran et al, in review), demonstrating that ventral plumage color is the target of sexual selection via mate choice. Here, I find that variation in juvenile plumage coloration is largely influenced by an individual's environment, with additive genetic variation having a minor impact (table 4.2). As juvenile plumage color predicts adult coloration (chapter 3), these results suggest that male coloration is

Table 4.3. Summary of variance component and ratio estimates (95% BCI) for the various color metrics estimated using multivariate animal model. Variance ratios were calculated as follows: narrow sense heritability ($h^2 = V_A/V_P$), the effect of early environment ($ee^2 = V_{EE}/V_P$), and the effect of rearing environment ($ee^2 = V_{RE}/V_P$).

	Theta			Phi			R _A			Brightness		
	Variance Component	Variance Ratio	Variance Component	Variance Component	Variance Ratio	Variance Component	Variance Component	Variance Ratio	Variance Component	Variance Ratio	Variance Component	Variance Ratio
Additive Genetic	0.015 (0.013 - 0.019)	0.163 (0.132 - 0.205)	0.016 (0.013 - 0.019)	0.161 (0.133 - 0.205)	0.017 (0.013 - 0.02)	0.165 (0.132 - 0.206)	0.016 (0.013 - 0.02)	0.172 (0.136 - 0.210)				
Nest of Origin	0.03 (0.024 - 0.043)	0.373 (0.281 - 0.421)	0.032 (0.024 - 0.044)	0.352 (0.281 - 0.427)	0.032 (0.025 - 0.045)	0.328 (0.282 - 0.426)	0.036 (0.024 - 0.044)	0.356 (0.271 - 0.418)				
Nest of Rearing	0.032 (0.023 - 0.042)	0.359 (0.274 - 0.416)	0.034 (0.024 - 0.044)	0.35 (0.269 - 0.413)	0.03 (0.024 - 0.044)	0.347 (0.273 - 0.417)	0.032 (0.024 - 0.043)	0.347 (0.266 - 0.410)				
Residual	0.012 (0.01 - 0.015)		0.014 (0.011 - 0.016)		0.013 (0.011 - 0.016)		0.013 (0.011 - 0.016)					
Total Phenotypic	0.095 (0.079 - 0.112)		0.093 (0.08 - 0.113)		0.1 (0.081 - 0.114)		0.098 (0.081 - 0.114)					

providing information about his environment during early development. Including sex in the model did not have a statistical effect; consequently, a female's environment during early development also affects her coloration later in life. In many passerine birds, both males and females participate in parental care such that a male's parental investment is large enough that mutual mate choice exists (Kokko & Johnstone 2002; Servedio & Lande 2006). While assortative mating by throat color has been shown in one North American population (Safran & McGraw 2004), there is no direct evidence that male barn swallows are exhibiting mate choice; however, female coloration does appear to provide information about individual quality (Safran & McGraw 2004) and physiology (Vitousek et al. 2013).

4.5.1 Mechanisms of Sexual Selection

Different mechanisms of sexual selection via mate choice make different predictions about whether variation in signal traits should be primarily genetically or environmentally influenced (table 4.1). By allocating paternity based on a plumage color trait that is primarily influenced by environmental variation, a female is likely gaining direct benefits by choosing to mate with a male expressing a particular phenotype (Iwasa & Pomiankowski 1999). However, given the long-term impacts of an individual's developmental environment (chapter 3), the information a female receives does not necessarily signal how a male is impacted by the current environmental context, but rather how his past environmental conditions shaped his phenotype. Consequently, females may use plumage color as a proxy to assess other traits that are impacted by developmental conditions. Developing in a favorable environment that promotes dark plumage development may also promote the development of behavior, physiology, and other morphology that could provide direct benefits to a female and her offspring. For example, a male with dark plumage could also be better at defending a territory from conspecific intruders or

predators, and his environment early in life impacted both traits. Other benefits provided by a male that experienced a favorable developmental environment include increased parental care (Kokko 1998; Iwasa & Pomiankowski 1999), reduced disease risk (Dufva & Allander 1995; Brawner et al. 2000), and reduced parasite exposure (Jacquin et al. 2011).

4.5.2 Environment

Overall, environmental variation explains a large proportion of phenotypic variation of nestling color (~ 67%). Given my experimental design of exchanging nestlings on day 2 of the nestling period, I explored the effects of early nest environment – a nestling’s original nest where it was incubated and cared for prior to exchange, and the effects of rearing environment – the nest where a nestling was cared for during the remainder of the nestling period. Both environments contribute to color variation (early environment: 35%; rearing environment: 32%; table 4.3).

The early environment would include some non-genetic maternal effects such as hormones deposited in the egg and incubation behavior. As shown in chapter 3, maternal effects explain a large proportion of phenotypic variation and that appears to be reflected here as well. However, because I had very few mothers with multiple broods in this experiment, I could not directly estimate the influence of non-genetic maternal effects. The early environment also includes conditions and parental behaviors experienced in the first two days post hatching. These effects explained the largest amount of phenotypic variation and I can speculate about what the specific factors might be. Temperature seems a likely factor as melanogenesis can be temperature dependent as seen in Siamese cats (Iljin & Iljin 1930) and other mammals (see Schmidt-Küntzel et al. 2005); perhaps incubation and early brood temperature are important factors in the production of melanin pigments to be deposited in feathers. Feather development

begins during incubation (Willier & Rawles 1940); therefore temperature effects would be seen in the early environment more so than the later rearing environment. Incubation and brood temperature are likely a result of both parental behavior and environmental conditions such as ambient temperature and nest location within a breeding site (e.g., proximity to door/window and presence of farm animals). Hormones deposited in eggs are another likely factor. In adult barn swallows, circulating carotenoid levels correlate with throat coloration (Safran et al. 2010a), and concentrations of carotenoids deposited in eggs by female barn swallows is variable (Safran et al. 2010b).

The rearing environment also explained a large proportion of color variation in nestling barn swallows. Temperature may continue to play a role in melanogenesis as nestlings continue to develop their juvenile plumage. Additional factors in the rearing environment include quality and quantity of food, parasite exposure, stress, and many others. While melanin-based coloration is typically not dependent on nutrition, it does require specific amino acids and the availability of these might vary based on diet (McGraw 2006a). Parasites, both endo- and ecto-parasites will illicit an immune response (Owen et al. 2010), which could have cascading effects on other physiological processes such as melanogenesis. Jenkins et al (2013) found no correlation between stress-induced corticosterone levels and color in male barn swallow nestlings, but the presences of stressors such as predators and competitors, as well as level of human disturbance might affect color development independently of stress hormones. Future work aimed at identifying specific early and rearing environmental factor that influence color development are needed.

Given the strong influence of environment on melanin-based color development, a female may select a particular nest (Safran 2007), rather than a particular social mate, to ensure that her

offspring experience a good developmental environment. In a favorable environment, she will produce offspring with darker plumage that will have the added advantages of better thermoregulation, higher resistance to feather-degrading bacteria and other ectoparasites, and plumage that is more resistant to wear (see Hubbard et al. 2010).

4.5.3 Heritability of melanin-based color

My results, both here and in chapter 3 of this dissertation, demonstrate that heritability of melanin-based coloration in North American barn swallows is approximately 16%, meaning that 16% of total phenotypic variation in melanin-based color is explained by additive genetic variation. This estimate is much lower than an estimate for the same trait in a different population of barn swallows (~ 80%: Saino et al. 2013). My study differs from this in that it pairs a powerful cross-foster experimental design with an analytical approach (animal model) that can take into account several sources of non-independence (including additive genetic variance); whereas a parent-offspring regression, unless paired with a careful experimental design, cannot control for environmental factors that can result in phenotypic similarities among relatives. Few studies have explored the heritability of quantitative variation in melanin-based coloration, but have instead focused on polymorphic color or variation in plumage patch size, traits that are typically highly heritable (> 70%) (Roulin & Dijkstra 2003; Bize et al. 2006; Gasparini et al. 2009; Quesada & Senar 2009).

4.6 Conclusions

Coloration in North American barn swallows is influenced by both genetic and environmental variation with environmental variation explaining a much larger proportion of phenotypic variation. As different mechanisms of sexual selection make different predictions

about relative roles of genetic and environment (table 4.1), I can infer that females use this colorful plumage trait in mate choice to gain primarily direct benefits. However, females also allocate more paternity to social mates with darker plumage (Safran et al. 2005; Safran et al, in review); consequently, females may use coloration to assess both direct *and* indirect benefits. Alternatively, females may select a nesting site (rather than a particular social mate) that will provide her offspring with a quality environment, and solicit extra-pair copulations from darker males, relative to her social mate, to provide quality genes to her offspring. Future studies should explore the true genetic fitness of darker vs. lighter males to better understand mate selection and the high occurrence of extra-pair mating in this population.

Chapter 5

Comparing G: Evidence for genetic divergence between two populations of barn swallows⁴

5.1 Abstract

Understanding phenotypic divergence among populations is a hallmark of research in evolutionary biology. To make predictions about the evolutionary forces driving phenotypic divergence, we need to know the underlying genetic variance for a divergent traits as well as the genetic covariance among traits. Estimating the genetic variance and covariance for multiple aspects of phenotype (G-matrix) within a species provides insight into how a trait will respond to selection. Comparing G-matrices among populations can provide information about evolutionary history and whether future selection will increase phenotypic divergence. Here, I compared G-matrices between phenotypically distinct subspecies of barn swallows in the Czech Republic (*H.r.rustica*) and North America (*H.r. erythrogaster*). I found that the genetic variance/covariance structure of a multivariate color trait differs between these populations indicating that selection on color will not produce similar evolutionary responses in these populations. Moreover, existing evidence suggest that divergent sexual selection on color and other aspects of phenotype has led to the current phenotypic divergence among these subspecies. Consequently, selection for different phenotypic optima may have led to both phenotypic and genetic divergence among these populations of barn swallows.

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5.2 Introduction

How and why populations diverge are fundamental questions in evolutionary biology. A major part of answering these questions involves understanding how populations will respond to the selective pressures they experience and how these selective pressures vary. As selection acts on an organism's entire phenotype, rather than a single trait in isolation, predicting how a population will respond to selection requires knowing the underlying genetic covariance among multiple aspects of phenotype (Lande 1979; Phillips & Arnold 1989; Arnold et al. 2008). The variance/covariance structure for a set of traits can differ among populations, and these differences can provide insight into the evolutionary processes that have influenced genetic and phenotypic variation within and among populations (Hohenlohe & Arnold 2008; Arnold et al. 2008; Aguirre et al. 2014) (table 5.1). Melanin-based coloration is a multivariate trait as it varies in both the total amount of pigment in a tissue as well as the ratio of pigment type (eumelanin vs. pheomelanin) resulting in quantitative color variation (McGraw 2006a; Hoekstra 2006; Hubbard et al. 2010). The correlation among pigment concentration and pigment type ratio can differ across populations and species such that similar selective pressures do not produce the same evolutionary response.

In the past 40 years, the field of quantitative genetics has experienced amazing growth (Walsh 2014). In particular, our understanding of the additive genetic variance/covariance structure among multiple traits (G matrix or G) has advanced dramatically. Darwin (1859) recognized the importance of covariance among traits and how that covariance could affect the response to selection seen in individual traits. More recently, simulation studies reveal that over long timescales, G responds to selection in predictable ways, especially when selection is aligned

Table 5.1. Possible evolutionary interpretations of a difference versus no difference in G when comparing populations with phenotypic divergence.

	Effects on G	Interpretation of G differences	Interpretation of G Similarities
Genetic Drift	Reduced variation along eigenvectors (size)	Random loss and fixation of alleles differs among small populations such that the overall genetic variation is reduced (size), but the correlation among traits (shape and orientation) remains constant.	Overall genetic variation is similar among populations despite random allelic differences.
Selection	Reduced variation along eigenvectors (size); change eigenvalues of eigenvectors (shape), change in relationship among eigenvectors (orientation)	Selection has reduced the overall genetic variation differently in each population (size); selection for one trait has reduced the variation along one axis of G differently in each population (shape); selection has broken down the correlation among traits (linkage/pleiotropy) in one population while it is maintained in another.	Selection has been strongest along the same axis of G such that the shape, size, and orientation of G is maintained, but the trait space occupied differs; Due to founder effects or past genetic drift, the presence of different alleles in each population produce different phenotypic effects while maintaining the size, shape, and orientation of G.

with the leading eigenvector, or the axis that explains the largest amount of variation, of G (Jones et al. 2003; Arnold et al. 2008). The genetic covariance among traits, as well as the genetic variance of individual traits, will affect the trajectory toward a fitness optimum on a fitness landscape, with the resulting path to the peak often being curved rather than direct (Lande 1979, 1980; Arnold et al. 2001). Consequently, the genetic covariance structure among multiple aspects of phenotype may constrain evolution within and among populations. For example, if two traits are correlated and have unequal genetic variance, directional selection acting on the trait with lower genetic variance will result in a correlated response to selection by the other trait, and prevent the first trait from moving perfectly in the direction of selection.

G can also be compared across species or populations. Empirical studies comparing G for the same set of traits among closely related populations have revealed that the structure of G is often conserved (Arnold et al. 2008). However, simulation and empirical studies show that evolutionary forces, such as drift, selection, and mutation can cause G to wobble, and sometimes change significantly (Phillips et al. 2001; Blows & Higgie 2003; Hunt et al. 2007; Hohenlohe & Arnold 2008; Arnold et al. 2008; Hine et al. 2011; Roff & Fairbairn 2012). Thus, when differences in G are detected, additional information about ancestral phenotypes or selection differences (direction and strength) is needed to determine which evolutionary force is more likely (Johansson et al. 2012). G can also be used to explore historical patterns of selection and drift within a group of species (Lande 1979; Hohenlohe & Arnold 2008; Arnold et al. 2008; Aguirre et al. 2014). Combining quantitative genetics and comparative phylogenetics, Hohenlohe and Arnold (2008) developed a model to test whether phenotypic divergence among related species can be best explained by neutral evolution (drift-mutation equilibrium) or selection;

however, this model assumes a uniform G across all species, an assumption that is not always met.

Barn swallows (*Hirundo rustica*) have a Holarctic distribution and there is quantitative divergence in multiple aspects of phenotype among the six subspecies (Safran et al, in review). Evidence suggests that divergent selection may have led to current phenotypic differences among these subspecies in several aspects of phenotype: ventral color, wing length, and the length of outer tail streamers (Saino et al. 1997; Safran et al. 2005; Vortman et al. 2013). To infer whether divergent selection underlies phenotype differences in color, I first constructed quantitative genetics models to examine the influences of the environment, genetics, and trait covariance on juvenile color, which is predictive of adult color (chapter 2), in a population of barn swallows from the Czech Republic (*H.r. rustica*). Next, I compared G, estimated using the same experimental design and the same model parameters, between these closely related populations (North America: *H.r. erythrogaster*, Chapter 4, and Czech Republic: *H.r. rustica*, reported here) to determine whether genetic variance differs in such a way that selection would cause similar or dissimilar phenotypic responses.

5.3 Methods

5.3.1 Study system

I studied two phenotypically distinct populations of barn swallows, one in North America (*H.r. erythrogaster*) and one in the Czech Republic (*H.r. rustica*). These subspecies differ in several aspects of ventral plumage coloration (see below) (figure 5.1; Adults: theta – $t_{380.64} = -18.457$, $p < 0.001$; phi – $t_{348.62} = 21.138$, $p < 0.001$; $r_A - t_{533.20} = 19.589$, $p < 0.001$; brightness – $t_{338.91} = -18.327$, $p < 0.001$; Nestlings: theta – $t_{531.65} = -22.046$, $p < 0.001$; phi – $t_{462.79} = 12.951$, p

< 0.001 ; $r_A - t_{431.36} = 15.898$, $p < 0.001$; $\text{brightness} - t_{368.27} = -17.802$, $p < 0.001$). In both populations, males and females form social pairs and raise one to two broods of nestlings – some pairs in the North American population successfully raise a third brood. Extra-pair young (EPY) are common in both populations, but the frequency of extra-pair young is higher in North America (47% of offspring are EPY) relative to the Czech Republic (16% EPY). The Czech population also has a significant amount of conspecific brood parasitism (9% parasitic young) with females laying an egg in another pairs' nest (Petrželková & Albrecht unpubl. data); if this occurs in the North American population, it is rare.

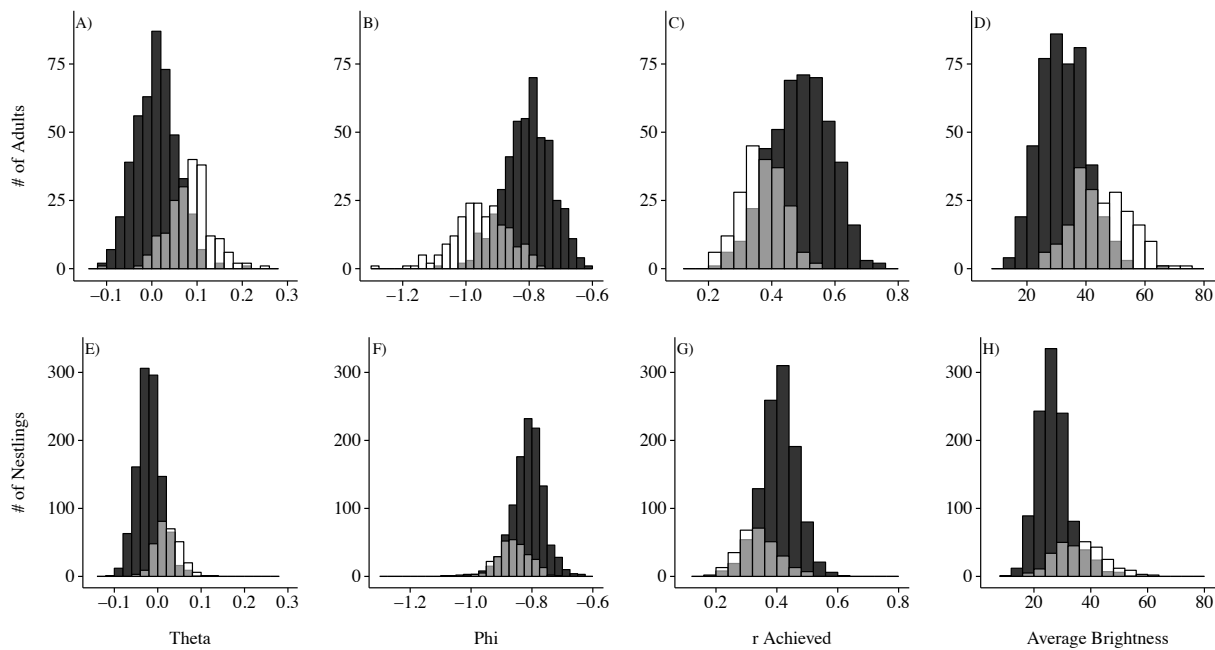


Figure 5.1. Population differences in color metrics in both adults (A-D) and nestlings (E-H). North America is in black, Czech Republic is in white, and where the distributions overlap, the bars are gray.

5.3.2 Cross-fostering experiment

I replicated the cross-fostering experiment detailed in chapter 4 in a breeding population in the Czech Republic (Southern Bohemia). I paired nests based on hatch day and brood size; I only included nests with four or more nestlings and nest pairs had the same brood size plus or

minus one nestling. On day two of the nestling period (with hatch day being day zero) I exchanged two nestlings that were matched for mass between paired nests (see fig. 4.1 for details). I banded nestlings on day 6, and took the mass and tarsus length of each nestling at day 6, day 9, and day 12 of the nestling period. Additionally, on day 12, I took additional measurements (wing length) and took blood and plumage samples (breast only) for paternity and color analyses (described in detail in chapter 3). Finally, I estimated when and how many nestlings fledged from successful nests. However, compared to the North American population (chapter 4), breeding sites were more densely occupied and fewer sites made up the population; consequently, many of the reciprocal exchanges took place between nests within the same breeding site (barn).

5.3.3 Paternity

Genotyping. Blood samples were dried and DNA was extracted and purified using the DNeasy® Blood and Tissue kit (Qiagen). Offspring and adults were genotyped at six high polymorphic microsatellite autosomal loci, which were previously developed for barn swallows: Hir6, Hir10, Hir15, Hir20, Hir22 (Tsyusko et al. 2007) and HrU10 (Primmer et al. 1995). The microsatellite loci were amplified in a single multiplex PCR using fluorescently labelled primers and a Multiplex PCR kit (Qiagen). The reaction conditions used were 15 min at 95 °C followed by 35 cycles of 30 s at 94°C, 90 s at 56 °C and 60 s at 72 °C, with a final extension of 30 min at 60 °C. PCR products were mixed with GeneScan™-500 Liz® Size Standard (Applied Biosystems) and formamide. These mixes were denatured for five min at 95 °C, cooled down on ice and analysed using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Binning was performed in program FlexiBin (Amos et al. 2006). Genotypes were then scored with the GeneMarker® version 1.9 software (Softgenetics). We calculated observed and

expected heterozygosity, the probability of exclusion and the frequency of null alleles for each locus using Cervus version 3.0.3 (Field Genetics Ltd; (Kalinowski et al. 2007)). The combined non-exclusion probability of the marker set was 6.33×10^{-3} for the first parent and 4.75×10^{-4} for the second parent. I used the program Micro-Checker to test for possible scoring errors due to allelic drop-outs or stuttering (Van Oosterhout et al. 2004).

In total, 1658 individuals from the Czech Republic population were genotyped at all six loci, 7 individuals were genotyped at five loci. Samples that were not genotyped at least at 5 loci were amplified in a PCR reaction again. Individuals that could not be genotyped at least at 5 loci were excluded from the analyses (2 females, 1 male and 1 offspring).

152 adult birds were genotyped more than once because of their breeding in multiple seasons. These repeated genotyping of the same birds was used to assess the probability of genotyping errors. 140 (92.1%) individuals were without any difference in particular genotypes (identical genotypes), 11 individuals (7.2%) differed at one locus and 1 bird (0.7%) differed at 2 loci. Overall, average genotyping error per locus was 0.006.

Parentage assignment. I used program Cervus 3.0 (Kalinowski et al. 2007) for parentage assignment. First, I carried out a maternity analysis in Cervus, i.e. log-likelihood statistics were computed for all possible offspring-candidate mother pairs (hereafter LOC). In nests where the social partner based was known based on field observation, male ID was included. The best candidate mother was considered to be a genetic mother of given chick in the case of Delta statistics (i.e. LOC difference between the most likely and second most likely mother) significance and full compatibility or one mismatch in offspring-putative mother genotype which could be ascribed to the presence of null alleles, allelic drop-outs (i.e. the putative female and

CBP offspring were heterozygotes at this locus) or a shift of 2-4 base pairs. Each offspring typically match only one mother on all loci.

In the next step, I carried out a paternity analysis with the known mother for the offspring whose maternity assignment was successful in the previous analysis. The most probable male was assigned to each young. Males were considered genetic fathers only if they had 0 or 1 mismatch and if the delta value was significant. As social fathers were assigned using behavioral observations (only males observed repeatedly feeding chicks were considered the social fathers of young in a particular nest; 206 nests, 84.1% of all nests) it was straightforward to distinguish within pair vs. extra pair paternities in these cases. If the identity of social male at a given nest was not known based on field observations (39 of all 245 nests) and if EPP did occur (11 of 39 nests), I classified the status of offspring and the social male of the putative nest as unknown.

In the last step, I carried out a parent-pair analysis with known sexes. This analysis confirmed the previous assignments based on the maternity and paternity analyses and in 3 cases found a genetic mother for chicks with an unassigned female after the first step.

Significance of parentage assignment was assessed based on the observed value of delta statistics (i.e. the LOC difference between best vs. second best candidate parent) with the critical value of delta (95% confidence the best parent correspond to the real genetic parent). The critical delta was computed independently each of Cervus analysis using 10,000 simulated cycles, the known distribution of allele frequencies (observed in our population) and genotyping errors 1%. We assumed that 80% of breeding females and 90% of males were sampled.

5.3.4 Color measurements

Plumage samples were taken from all nestlings on day 12 of the nestling period. Plumage color was measured with a spectrometer (USB 4000, Ocean Optics), pulsed xenon light (PX-2,

Ocean optics) and SpectraSuite software (v2.0.151) and quantified in tetrahedral color space (Stoddard & Prum 2008) using the R package ‘pavo’ (Maia et al. 2013). Using this method, four color metrics are extracted that relate to either the ratio of eumelanin and pheomelanin pigments: theta and phi are angle measurements that dictate where a sample is found in the color space and provide information about a sample’s hue, or the total concentration of melanin pigments: r is a measure of saturation and as r varies with the color space I used r achieved (r_A), which is a measure of r relative to the maximum value for that color space and brightness is an achromatic measure of light reflectance. All color metrics are highly repeatable (chapter 3).

5.3.5 Quantitative genetics

Genetic and Environmental influences on color in the Czech Republic. I used an animal model to estimate key variance components and the proportion of phenotypic variance explained by each component. Analytic methods used here to estimate these components for the Czech Republic population reflect those used in chapter 4 for the North American population.

Table 5.2. Posterior mode (and 95% BCI) of all fixed effects included in maximal model (DIC: -3216.179). None of the fixed effects had a statistical effect (posterior distributions overlap zero) and were therefore not included in the final models.

	Posterior Mode (95% BCI)
	0.0001
Sampling Date	(-0.002 – 0.002)
	-0.002
Sex	(-0.026 – 0.020)
	-0.00003
Right Wing Length	(-0.003 – 0.003)

Sex, date, and right wing length were initially included as fixed effects, however, as they had no statistical effect on the model, they were excluded from the final model (table 5.2). For each color metric, I partitioned variance into additive genetic variance, early nest environment, and rearing nest environment variance by including the following random effects in the model: pedigree, nest of origin and nest of rearing. The total phenotypic variance (V_P) was calculated as the sum of the variance components: $V_P = V_A + V_{EE} + V_{RE} + V_R$. Where V_A is the additive

genetic variance, V_{EE} is the early nest environment variance, V_{RE} is the rearing environment variance, and V_R is the residual variance (Falconer & Mackay 1996; Lynch & Walsh 1998). Using these variance components, we calculated narrow sense heritability ($h^2 = V_A/V_P$), the effect of early environment ($ee^2 = V_{EE}/V_P$), and the effect of rearing environment ($re^2 = V_{RE}/V_P$).

I specified the priors for the variance-covariance matrix as an inverse Wishart matrix distribution (de Villemereuil 2012). All models were run for 502,00 iterations, with a burn in of 2,000 iterations, and every 200th iteration was stored (autocorrelations were weaker than 0.063 for all variance components) with an effective sample size of 2500 MCMC samples.

Table 5.3. G matrices for North America and the Czech Republic showing the genetic variance (along diagonal) and covariance (off diagonal) with 95% Bayesian Credible Intervals for each color metric.

North America				
	Theta	Phi	r_A	Brightness
Theta	0.01594 (0.01263 - 0.01885)	-0.00018 (-0.00222 - 0.00238)	-0.00018 (-0.0028 - 0.00168)	-0.00013 (-0.00213 - 0.00234)
Phi		0.01631 (0.01308 - 0.01964)	0 (-0.00208 - 0.00252)	-0.00018 (-0.00283 - 0.00175)
r_A			0.01645 (0.01279 - 0.01962)	-0.00112 (-0.00325 - 0.00162)
Brightness				0.016 (0.0133 - 0.01983)
Czech Republic				
	Theta	Phi	r_A	Brightness
Theta	0.02158 (0.01744 - 0.02863)	-0.00038 (-0.00461 - 0.00319)	0.00035 (-0.00453 - 0.00347)	0.00069 (-0.00386 - 0.00496)
Phi		0.0233 (0.01844 - 0.02967)	-0.00054 (-0.00335 - 0.00471)	-0.00042 (-0.0058 - 0.00318)
r_A			0.02325 (0.0183 - 0.03016)	-0.00235 (-0.00571 - 0.00307)
Brightness				0.02532 (0.02024 - 0.03303)

Extracting G. From the results of the animal model in North America (chapter 4) and the Czech Republic (this chapter), I extracted the G matrices (95% BCI) (table 5.3). Given the model parameters used to estimate variance components, model results for each population yielded 2500 MCMC samples for each variance component (trait variances and pairwise trait covariances). These values are then arranged into a single G array that includes population information in order to compare the structure of G for these two populations (described below).

Comparing G. I compared G of North America and Czech Republic barn swallow color using two methods: random skewers (Cheverud & Marroig 2007; Aguirre et al. 2014) and genetic covariance tensor (Hine et al. 2009; Aguirre et al. 2014). Both methods explore differences in genetic variance for different trait combinations among populations, however the genetic covariance tensor method is more complex in that it considers differences in genetic variance across the entire phenotypic space, whereas the random skewers method, which thus far has been more widely used, randomly probes phenotypic space to detect differences (Aguirre et al. 2014).

The random skewers method is used to estimate the difference in the magnitude of genetic variances among populations by comparing the simulated responses of populations to random selection vectors or skewers (here I use 1000 skewers) (Cheverud & Marroig 2007; Aguirre et al. 2014). Differences in the response to selection suggest the orientation (directionality of eigenvectors)

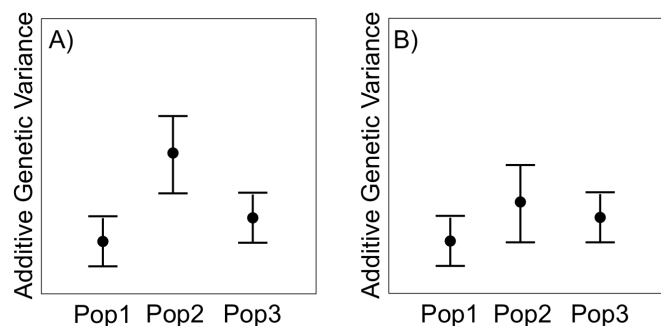


Figure 5.2. Possible outcomes of the projections of R. A) shows a scenario where an eigenvector of R has significant differences in genetic variance among populations. B) shows an eigenvector of R that does not have significant differences. When differences are detected, the loadings of the eigenvector in R reveal trait combinations that explain the differences in genetic variance.

of G differs among populations (Cheverud 1996; Cheverud & Marroig 2007; Hansen & Houle 2008; Aguirre et al. 2014). Once differences are detected, I can identify the phenotypic space that differs by looking at the eigenstructure of the R -matrix – a matrix that represents the product-moment G of the vector elements calculated using the skewers that differ among populations. Projecting R on the set of observed G (from North America and the Czech republic) shows which eigenvectors (and therefore which trait combinations) of R result in non-overlapping intervals of genetic variances between populations (figure 5.2). Using this method, I can estimate the degree of similarity between G in North America and G in Europe (Cheverud & Marroig 2007), and identify the trait combinations along the dissimilar eigenvectors that explain the largest amount of variation.

The genetic covariance tensor method compares the difference matrix of the two populations to a randomized difference matrix to determine how the matrices differ (figure 5.3) (Hine et al. 2009; Aguirre et al. 2014). In multilinear algebra, tensors describe relationships in multivariate space; vectors are first order tensors, matrices are second order tensors. Higher

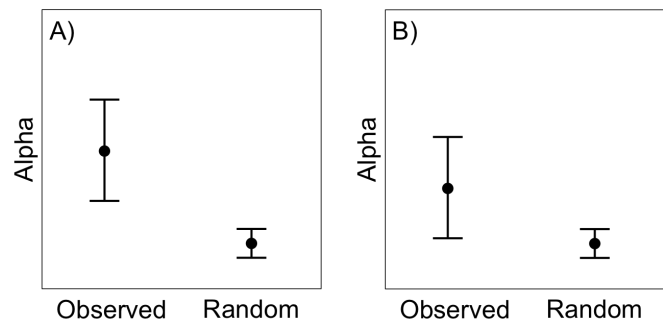


Figure 5.3. Possible outcomes of the comparing the observed difference matrix to a randomized difference matrix. A) shows a scenario where alpha does not overlap and the populations differ in genetic variance. B) shows a scenario where alpha overlaps and there is no evidence of divergence.

order tensors can be used to characterize lower-order variables (Hine et al. 2009). The G matrix, a second-order tensor, summarizes the variances and covariances within a population. To summarize the differences among multiple G -matrices for different populations requires a fourth-order covariance tensor, which can be described with a set of eigenvalues and second-

order eigentensors (Hine et al. 2009). From the eigentensors, I can identify combinations of traits in the entire phenotypic space that have diverged in genetic variance.

Aguirre et al (2014) argue that while these two methods both identify trait combinations that differ in genetic variance, the genetic covariance tensor method is more efficient and direct for comparing differences in G among populations, but few studies have used this method to date. All animal models were fit with the MCMCglmm package in R (Hadfield 2010). Matrix comparisons were performed using functions designed by Aguirre *et al* (2014). All other analyses were performed in R v3.0.3 (Team 2014).

Table 5.4. Posterior mode of variance components for each color metric estimated from the animal model. V_A is additive genetic variance, V_{RE} is variance due to rearing environment, V_{EE} is variance due to early environment, V_R is residual variance, and V_P is total phenotypic variance.

	Theta (95%BCI)	Phi (95%BCI)	R_A (95%BCI)	Bright (95%BCI)
V_A	0.022 (0.017 - 0.029)	0.023 (0.018 - 0.03)	0.023 (0.018 - 0.03)	0.025 (0.02 - 0.033)
V_{RE}	0.051 (0.034 - 0.079)	0.053 (0.037 - 0.081)	0.048 (0.037 - 0.08)	0.056 (0.036 - 0.081)
V_{EE}	0.052 (0.036 - 0.079)	0.051 (0.036 - 0.08)	0.054 (0.037 - 0.084)	0.057 (0.036 - 0.083)
V_R	0.017 (0.014 - 0.022)	0.018 (0.015 - 0.023)	0.018 (0.014 - 0.023)	0.019 (0.016 - 0.026)
V_P	0.155 (0.12 - 0.192)	0.149 (0.121 - 0.193)	0.154 (0.123 - 0.197)	0.151 (0.129 - 0.205)

5.4 Results

5.4.1 Genetic and Environmental influences on color in the Czech Republic. Results from the animal model demonstrate that variation in ventral plumage coloration in European barn swallows is strongly explained by environmental variation, similar to color variation in the North

American subspecies (table 5.4 and first column of table 5.5). As in North American barn swallows (chapter 4, this dissertation), ventral plumage color in European barn swallows has a heritability of 0.15 to 0.16 (15-16% of phenotypic variation explained by additive genetic variation), whereas environmental variation explains approximately 65 to 70% of phenotypic variation (table 5.5, column 1). I found no significant genetic correlations among color metrics (table 5.3), and the overall results were relatively insensitive to the priors specified for the variance/covariance structure (table 5.5).

Table 5.5. Summary of model outcomes using various priors; model outcomes were relatively insensitive to the prior parameterization.

		Final	Gamma = 0.5	Gamma = 0.1	Gamma = 0.01
DIC		-1082.444	-1770.164	-3092.836	-4081.286
Theta	h²	0.149 (0.112 - 0.195)	0.153 (0.109 - 0.196)	0.159 (0.117 - 0.211)	0.195 (0.141 - 0.285)
	re²	0.354 (0.262 - 0.461)	0.353 (0.267 - 0.462)	0.363 (0.252 - 0.448)	0.279 (0.191 - 0.369)
	ee²	0.361 (0.27 - 0.463)	0.344 (0.273 - 0.457)	0.346 (0.261 - 0.449)	0.274 (0.201 - 0.386)
Phi	h²	0.153 (0.111 - 0.197)	0.155 (0.112 - 0.205)	0.169 (0.128 - 0.23)	0.202 (0.128 - 0.312)
	re²	0.367 (0.262 - 0.46)	0.365 (0.253 - 0.45)	0.318 (0.244 - 0.449)	0.259 (0.182 - 0.405)
	ee²	0.383 (0.267 - 0.464)	0.337 (0.263 - 0.463)	0.333 (0.239 - 0.44)	0.222 (0.14 - 0.339)
R_A	h²	0.149 (0.107 - 0.198)	0.158 (0.113 - 0.2)	0.186 (0.13 - 0.234)	0.216 (0.133 - 0.34)
	re²	0.352 (0.255 - 0.459)	0.35 (0.261 - 0.465)	0.34 (0.232 - 0.442)	0.263 (0.157 - 0.396)
	ee²	0.359 (0.263 - 0.471)	0.371 (0.266 - 0.467)	0.338 (0.242 - 0.444)	0.214 (0.14 - 0.371)
Brightness	h²	0.161 (0.115 - 0.207)	0.165 (0.126 - 0.225)	0.216 (0.156 - 0.293)	0.173 (0.109 - 0.361)
	re²	0.318 (0.248 - 0.449)	0.324 (0.243 - 0.435)	0.284 (0.199 - 0.384)	0.175 (0.095 - 0.267)
	ee²	0.364 (0.266 - 0.467)	0.335 (0.257 - 0.449)	0.274 (0.204 - 0.39)	0.158 (0.089 - 0.248)

5.4.2 Comparing G.

Random Skewers Method. Projecting

1000 random selection vectors through G for North America and Czech Republic yields 55 skewers with posterior distributions (95% HPD intervals) of the genetic variance in their direction that do not overlap, whereas the same projection with a randomized G matrices (i.e., unstructured

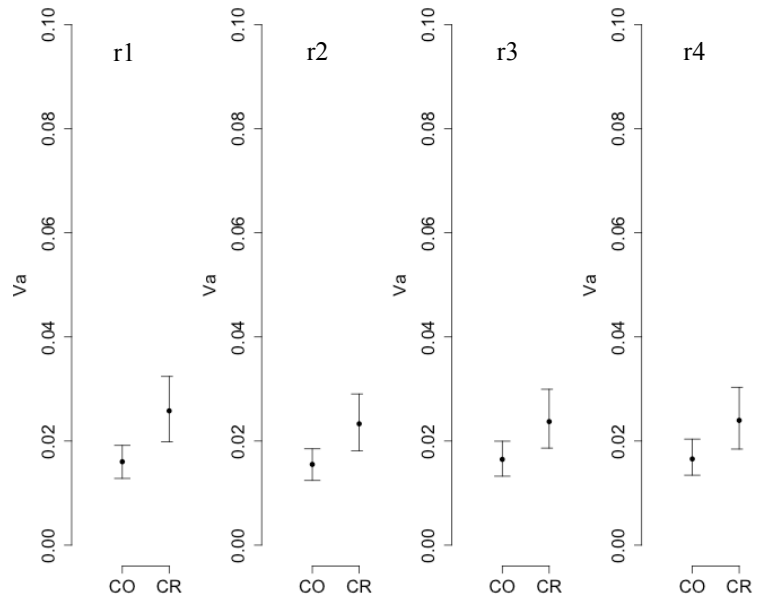


Figure 5.2. Genetic variance in the direction of each of the eigenvectors of R for each population. Only the first eigenvector of R results in significant differences

populations) found no skewers with HPD intervals that do not overlap. These 55 vectors summarize the directions in multivariate space where matrices differ significantly in variance. From these, I determined which parts of phenotypic space illustrate significant differences in genetic variance (table 5.6) by looking at the eigenstructure of the R-matrix. The projection of R on the set of observed G revealed that only the first eigenvector of R resulted in non-overlapping intervals of genetic variances between North America and Czech Republic (table 5.7, figure 5.2). Ventral color brightness loads heavily on this eigenvector (table 5.6) demonstrating that genetic variance for brightness dictates the difference in G between North America and Czech Republic.

Table 5.6. Eigenstructure of the R matrix from the random skewer method, which describes which parts of the phenotypic space tend to show significant differences in genetic variance.

	Eigenvector 1	Eigenvector 2	Eigenvector 3	Eigenvector 4
Eigenvalue	0.724	0.180	0.068	0.045
Theta	0.304	-0.586	0.517	0.545
Phi	-0.021	0.670	0.742	0.027
R _A	0.208	-0.356	0.358	-0.838
Brightness	0.929	0.286	-0.233	0.010

Genetic Covariance Tensor Method. With only two populations (North America and Czech Republic) the genetic covariance tensor will have at most, one non-zero eigenvalue. This eigentensor describes a significant amount of genetic variance (figure 5.3). The eigenanalysis of this eigentensor shows that the leading eigenvector accounts for 32% of the variation in this eigentensor, and as seen in the random skewers method, brightness loads heavily on this eigenvector suggesting variation in brightness is driving differences in G among these populations (table 5.8). Projecting the first eigenvector of the eigentensor demonstrates that the Czech Republic population explains more genetic variance captured by this eigenvector compared to North America (figure 5.4).

Table 5.7. Only the first eigenvector of R is significantly different between populations.

	North America	Czech Republic
r1	0.016 (0.013 - 0.019)	0.026 (0.020 - 0.032)
r2	0.016 (0.012 - 0.019)	0.023 (0.018 - 0.029)
r3	0.016 (0.013 - 0.020)	0.024 (0.019 - 0.030)
r4	0.017 (0.013 - 0.020)	0.024 (0.018 - 0.030)

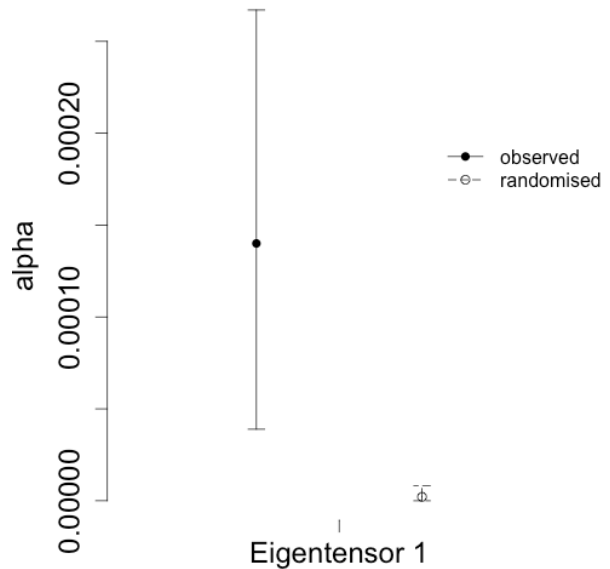


Figure 5.3. Eigenvalues of the non-zero eigentensor for posterior mean S, and 95% HPD interval for each MCMC sample of the observed and randomized G arrays.

Table 5.8. Eigenstructure of the first eigentensor from the genetic covariance tensor method.

	Eigenvector 1	Eigenvector 2	Eigenvector 3	Eigenvector 4
Eigenvalue	0.631	0.476	0.463	0.402
Theta	0.233	-0.555	0.332	0.726
Phi	-0.194	0.755	0.030	0.625
R _A	-0.188	0.134	0.936	-0.265
Brightness	0.934	0.322	0.111	-0.105

5.6 Discussion

Barn swallows in North America and the Czech Republic are phenotypically divergent with respect to several aspects of phenotype (see figure 5.1). Moreover, these traits appear to currently experience different selective pressures; manipulative studies demonstrate that higher within-pair paternity is causally linked with different traits in different populations (Saino et al. 1997; Safran et al. 2005; Vortman et al. 2013). In North America, males with darker plumage are allocated more paternity from their social mate (Safran et al. 2005; Safran et al, in review); in Israel, males with darker plumage *and* elongated tail streamers achieve the highest within pair paternity (Vortman et al. 2013); and preliminary results from the Czech Republic corroborate findings in Europe that males with elongated tails have higher within-pair paternity (Albrecht unpubl. data; Saino et al. 1997).

Results from this study demonstrate that selection on coloration would have different evolutionary responses in the two subspecies (*H.r. rustica* and *H.r. erythrogaster*) as both analytical methods reveal divergence in the genetic variance/covariance structure of plumage color between these populations. Furthermore, experimental evidence suggests divergent sexual selection among barn swallow subspecies was a driving force in population divergence (Safran et al. 2005; Vortman et al. 2013; Safran et al, in review); Consequently, I can infer that selection

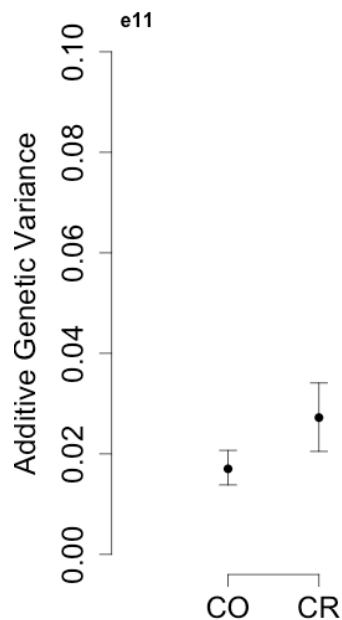


Figure 5.4. Posterior mean and the 95% HPD interval for the genetic variance along the direction of e11 (the first eigenvector of the eigentensor) for each population.

caused the differences in G shown here; however, I cannot definitively rule out genetic drift, which can affect the size of G (Phillips et al. 2001; Arnold et al. 2008; Aguirre et al. 2014). Stabilizing selection toward different adaptive peaks would lead to divergence in G creating stable G-matrices in both populations (Arnold et al. 2008; Aguirre et al. 2014); similarly, selection in North America, and no selection in Czech Republic could also lead to divergence in G (table 5.8). Cano et al (2004) demonstrated differences in G among two populations of *Rana temporaria*, but more importantly, they demonstrated that the shape and orientation of G changed in response to experimental selection in both populations.

Another explanation for divergence in G would be that there are different pools of alleles in each population, and the alleles present vary in effect size on the phenotype (table 5.1) (Arnold et al. 2008; Aguirre et al. 2014). In a simplified scenario, we can imagine that there are five genes involved in the development of plumage coloration. In population one, the alleles at those loci have large additive effects on multiple measures of color, and in population two, some of the effects are weaker. This will influence the eigenstructure of G and how coloration will respond to selection in both populations. In this scenario, the two populations would take different trajectories to reach the same adaptive peak. Alternatively, the alleles could have similar effect sizes such that the shape of G is similar, but phenotypic distributions resulting from the additive genetic variation of those alleles do not overlap.

While the two populations differ in all four measures of color (figure 5.1), genetic variance for achromatic brightness appears to be the driving factor in the divergence of G (table 5.5). As there is no significant genetic correlation among color metrics in either population (table 5.3), it is not surprising that a single color metric explains variation in G. Moreover, experiments that manipulate primarily brightness of plumage have demonstrated that, in North America,

females prefer males with the darkest plumage. While female preference for color is currently unknown in the Czech Republic population, results from this study suggest that selection on plumage brightness has been stronger in North America as genetic variance for brightness is reduced in North America relative to the Czech Republic.

Melanin-based plumage color is the result of both the type of melanin pigment (eumelanin vs. pheomelanin) and the total amount of pigment deposited into a feather (McGraw 2006a). Variation in brightness is primarily affected by the total amount of pigment deposited into a feather, which will affect how much light an individual's plumage reflects (or absorbs). Known pigmentation genes either affect the ratio of pigment type or the total amount of pigment deposited (Hubbard et al. 2010). Results from this study suggest that North American and European populations of barn swallows primarily demonstrate divergence in genes that influence pigment concentration, while genes that affect the ratio of pigment type are likely conserved across populations.

5.7 Conclusion

Selection acts on the genetics of populations through phenotypic variation. Consequently, understanding the underlying genetic variance for phenotypes provides information about how a population will respond to selection. Furthermore, by demonstrating how various aspects of phenotype are linked allows for more accurate predictions about evolutionary trajectories for a given population to be made. As I have shown here, estimating the genetic variance/covariance structure for a multivariate trait, and how it differs in divergent populations can be used to predict whether selection will cause further phenotypic divergence, and infer how past selection has shaped current phenotypic divergence. The methods for estimating G presented here, paired

with estimates of selection, ancestral phenotypes, or phylogenetic hypotheses can be used to infer evolutionary history for a species or species complex, which will further our understanding of evolutionary processes and population divergence.

Chapter 6

Summary and Conclusions

6.1 Summary of key findings

Colorful signals have long been known to be an important aspect of an animal's phenotype and used in many different contexts. This dissertation offers novel insight into the underlying mechanisms that affect melanin-based color development. Melanin-based coloration in vertebrates has often been studied in the context of color polymorphisms, and these discrete phenotypes have repeatedly been shown to strongly associate with simple genetic changes (Theron et al. 2001; Hoekstra & Nachman 2003; Mundy 2005; Nadeau et al. 2007; Uy et al. 2009; Mullen et al. 2009). My research on the underlying genetic variation for continuous melanin-based coloration tells a different story. I found that in two populations of barn swallows (*H.r. erythrogaster* and *H.r. rustica*) environmental variation explained the largest proportion of phenotypic variation (chapters 4 and 5). I also found that these environmental effects have long-term consequences on the development of the adult signal, as juvenile color is strongly predictive of adult color within an individual (chapter 3). This suggests that there is some developmental plasticity in melanogenesis and that the nest environment is a key component of adult signal development.

6.2 Future Directions

This research has led to many more questions about melanin-based coloration within and across populations of barn swallows. One of the most unexpected results of my dissertation research is that melanin-based coloration is strongly influenced by environmental variation. Consequently, a major question to address in the future is what specific factors are impacting

color development. I will continue to collaborate with another graduate student, Amanda Hund, to explore the role of parasites in color development. Blood feeding mites colonize barn swallow nests and there is extreme variation in infestation across nests; these mites are known to affect survival in barn swallows (Hund, unpubl. data). Our cross-fostering experiments have also shown that coloration of male nestlings is affected by mite exposure. In the future, we will explore other environmental variables, such as temperature, clutch size, and parental care. In particular, temperature during incubation and brooding being may be a key factor influencing melanogenesis.

I would also like to add additional subspecies to the analyses in chapter 5 to provide additional information about the divergence of this species complex. Also in collaboration with Amanda, I will be traveling to Israel to collect nestling color data and paternity for the *H.r. transitiva* subspecies. Results from chapters 3 and 4 demonstrate that full-sib/half-sib design created by high extra-pair paternity (EPP) in the populations yields similar quantitative genetic results, suggesting that in populations with high EPP, cross-fostering may not be necessary. As the Israel population has comparable rates of EPP, I can estimate heritability and environmental effects in the framework presented here without the cross-fostering experimental design. Ideally, I would compile these data from all six subspecies and put this into a phylogenetic framework to provide a better understanding of how melanin-based coloration has contributed to the divergence of this young species complex.

I have collaborated with an undergraduate student, Ryan Higgins, in trying to identify allelic variation in known pigmentation genes within and across populations that might explain phenotypic variation in barn swallow coloration. Preliminary results suggest there might be genetic differences in the Tyrosinase alleles present in *H.r. erythrogaster* and *H.r. rustica* (fig.

6.1). I would like to expand this work to include additional known pigmentation genes and additional populations. Exploring the specific pigmentation genes contributing to phenotypic divergence, and focusing on those that influence pigment concentration (which will primarily affect brightness), will provide further insight into the underlying mechanisms of divergence among barn swallows.

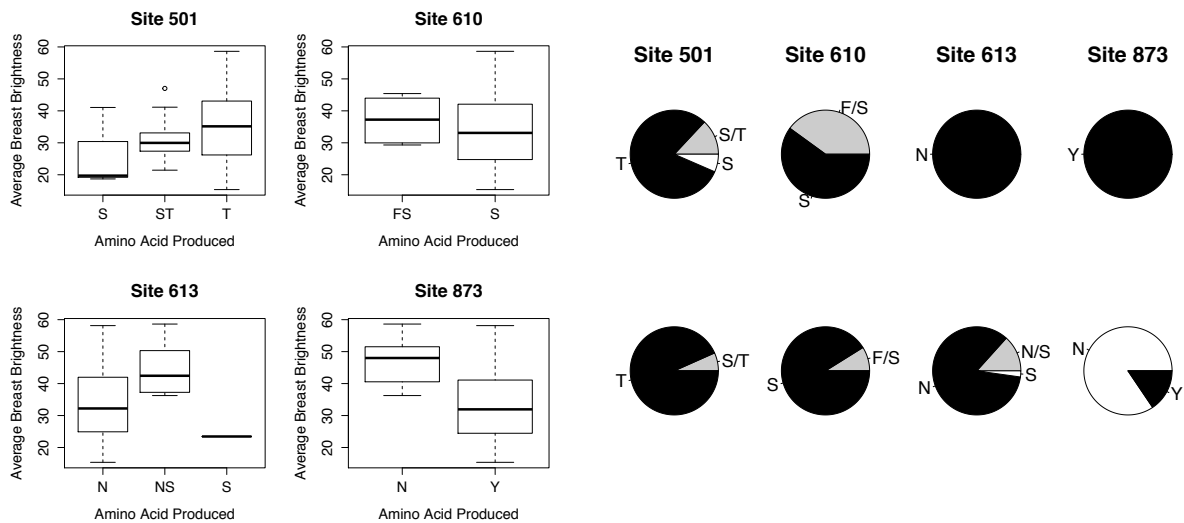


Figure 6.1. Boxplots showing that allelic variation in the Tyrosinase gene (pooled across *H.r.rustica* and *H.r. erythrogaster*) at four single-nucleotide polymorphisms (SNPs); three of the four SNPs (501, 613, and 873) appear to associate with coloration. The pie charts show allelic frequency in the two subspecies with the top row showing *H.r. erythrogaster* and the bottom row showing *H.r.rustica*.

6.6 Conclusions

Quantitative genetics is a rapidly growing field and new tools and methods continue to be developed. My dissertation research used some of these tools to demonstrate that a melanin-based plumage trait is influenced by the environment despite extensive evidence that melanin-based colors are typically under strong genetic control in other systems. I also employed comparative quantitative genetic tools to compare G-matrices across populations. While methods to compare matrices have been used for years, only recently have some comparative methods

been developed with an evolutionary framework in mind allowing for hypotheses about selection and drift to be tested (Hohenlohe & Arnold 2008; Aguirre et al. 2014). I hope that my work on quantitative genetics within and across populations of barn swallows will be the foundation for future studies aimed at understanding the factors that affect trait variation and the implication those factors have on evolution and species divergence.

7. References

- Aguirre, J. D., Hine, E., McGuigan, K. & Blows, M. W.** 2014. Comparing G: multivariate analysis of genetic variation in multiple populations. *Heredity*, **112**, 21–29.
- Amos, W., Hoffman, J. I., Frodsham, a., Zhang, L., Best, S. & Hill, a. V. S.** 2006. Automated binning of microsatellite alleles: problems and solutions. *Molecular Ecology Notes*, **7**, 10–14.
- Amundsen, T. & Pärn, H.** 2006. Female coloration: Review of functional and nonfunctional hypotheses. In: *Bird Coloration: Function and Evolution*, (Ed. by G. E. Hill & K. J. McGraw), pp. 280–345. Cambridge: Harvard University Press.
- Anderson, T. M., Von Holdt, B. M., Candille, S. I., Musiani, M., Greco, C., Stahler, D. R., Smith, D. W., Padhukasahasram, B., Randi, E., Leonard, J. a, Bustamante, C. D., Ostrander, E. A., Tang, H., Wayne, R. K. & Barsh, G. S.** 2009. Molecular and evolutionary history of melanism in North American gray wolves. *Science*, **323**, 1339–1343.
- Andersson, M. B.** 1994. *Sexual Selection*. Princeton: Princeton University Press.
- Arnold, S. J., Pfrender, M. E. & Jones, A. G.** 2001. The adaptive landscape as a conceptual bridge between micro- and macroevolution. *Genetica*, **112-113**, 9–32.
- Arnold, S. J., Bürger, R., Hohenlohe, P. a, Ajie, B. C. & Jones, A. G.** 2008. Understanding the evolution and stability of the G-matrix. *Evolution*, **62**, 2451–2461.
- Barrett, R. D. H., Rogers, S. M. & Schluter, D.** 2008. Natural selection on a major armor gene in threespine stickleback. *Science*, **322**, 255–257.
- Basolo, A. L.** 2006. Genetic linkage and color polymorphism in the southern platyfish (*Xiphophorus maculatus*): a model system for studies of color pattern evolution. *Zebrafish*, **3**, 65–83.
- Beamonte-Barrientos, R., Velando, A., Drummond, H. & Torres, R.** 2010. Senescence of maternal effects: aging influences egg quality and rearing capacities of a long-lived bird. *The American Naturalist*, **175**, 469–480.
- Bensch, S., Price, T. & Kohn, J.** 1997. Isolation and characterization of microsatellite loci in a *Phylloscopus* warbler. *Molecular Ecology*, **6**, 91–92.
- Biswas, S. & Akey, J. M.** 2006. Genomic insights into positive selection. *Trends in Genetics*, **22**, 437–446.
- Bize, P., Gasparini, J., Klopfenstein, A., Altwegg, R. & Roulin, A.** 2006. Melanin-based coloration is a nondirectionally selected sex-specific signal of offspring development in the alpine swift. *Evolution*, **60**, 2370–2380.

- Blows, M. W. & Higgie, M.** 2003. Genetic constraints on the evolution of mate recognition under natural selection. *The American Naturalist*, **161**, 240–253.
- Bolund, E., Schielzeth, H. & Forstmeier, W.** 2011. Correlates of male fitness in captive zebra finches - a comparison of methods to disentangle genetic and environmental effects. *BMC Evolutionary Biology*, **11**, 327.
- Bonduriansky, R.** 2007. Sexual selection and allometry: a critical reappraisal of the evidence and ideas. *Evolution*, **61**, 838–849.
- Brawner, W. R., Hill, G. E. & Sundermann, C. A.** 2000. Effects of coccidial and mycoplasmal infections on carotenoid-based plumage pigmentation in male house finches. *The Auk*, **117**, 952–963.
- Buchanan, K. L., Spencer, K. A., Goldsmith, A. R. & Catchpole, C. K.** 2003. Song as an honest signal of past developmental stress in the European starling (*Sturnus vulgaris*). *Proceedings of the Royal Society B: Biological Sciences*, **270**, 1149–1156.
- Cano, J. M., Laurila, A., Palo, J. & Merilä, J.** 2004. Population Differentiation in G Matrix Structure Due to Natural Selection in *Rana temporaria*. *Evolution*, **58**, 2013–2020.
- Cassey, P., Ewen, J. G., Marshall, N. J., Vorobyev, M., Blackburn, T. M. & Hauber, M. E.** 2009. Are avian eggshell colours effective intraspecific communication signals in the Muscipoidea? A perceptual modelling approach. *Ibis*, **151**, 689–698.
- Cerdá-Reverter, J. M., Haitina, T., Schiöth, H. B. & Peter, R. E.** 2005. Gene structure of the goldfish agouti-signaling protein: a putative role in the dorsal-ventral pigment pattern of fish. *Endocrinology*, **146**, 1597–1610.
- Cheverud, J. M.** 1996. Quantitative genetic analysis of cranial morphology cotton-top (*Saguinus oedipus*) and saddle-back (*S. fuscicollis*) tamarins. *Journal of Evolutionary Biology*, **9**, 5–42.
- Cheverud, J. M. & Marroig, G.** 2007. Comparing covariance matrices : Random skewers method compared to the common principal components model. *Genetics and Molecular Biology*, **30**, 461–469.
- Chevron, Z. A., Hackett, S. J. & Brumfield, R. T.** 2006. Sequence variation in the coding region of the melanocortin-1 receptor gene (MC1R) is not associated with plumage variation in the blue-crowned manakin (*Lepidothrix coronata*). *Proceedings of the Royal Society B: Biological Sciences*, **273**, 1613–1618.
- Cuthill, I. C., Bennett, A. T. D., Partridge, J. C. & Maier, E. J.** 1999. Plumage Reflectance and the Objective Assessment of Avian Sexual Dichromatism. *The American Naturalist*, **153**, 183–200.

- Danchin, E.** 2013. Avatars of information: towards an inclusive evolutionary synthesis. *Trends in Ecology & Evolution*, **28**, 351–358.
- Darwin, C.** 1859. *On the origin of species by means of natural selection*. 1st edn. London: John Murray.
- De Villemereuil, P.** 2012. Tutorial Estimation of a biological trait heritability using the animal model How to use the MCMCglmm R package.
- Dor, R., Safran, R. J., Sheldon, F. H., Winkler, D. W. & Lovette, I. J.** 2010. Phylogeny of the genus *Hirundo* and the Barn Swallow subspecies complex. *Molecular Phylogenetics and Evolution*, **56**, 409–418.
- Doucet, S. M., Shawkey, M. D., Rathburn, M. K., Mays, H. L. J. & Montgomerie, R.** 2004. Concordant evolution of plumage colour, feather microstructure and a melanocortin receptor gene between mainland and island populations of a fairy-wren. *Proceedings of the Royal Society B: Biological Sciences*, **271**, 1663–1670.
- Dufva, R. & Allander, K.** 1995. Intraspecific variation in plumage coloration reflects immune response in great tit (*Parus major*) males. *Functional Ecology*, **9**, 785–789.
- Eizirik, E., Yuhki, N., Johnson, W. E., Menotti-Raymond, M., Hannah, S. S. & O'Brien, S. J.** 2003. Molecular genetics and evolution of melanism in the cat family. *Current Biology*, **13**, 448–453.
- Emlen, D. J.** 1994. Environmental Control of Horn Length Dimorphism in the Beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Proceedings of the Royal Society B: Biological Sciences*, **256**, 131–136.
- Endler, J. A.** 1991. Variation in the appearance of guppy color patterns to guppies and their predators under different visual conditions. *Vision Research*, **31**, 587–608.
- Endler, J. A.** 1993. The color of light in forests and its implications. *Ecological Monographs*, **63**, 1–27.
- Endler, J. A. & Thery, M.** 1996. Interacting effects of lek placement, display behavior, ambient light, and color patterns in three neotropical forest-dwelling birds. *The American Naturalist*, **148**, 421–452.
- Endler, J. A., Westcott, D. A., Madden, J. R. & Robson, T.** 2005. Animal visual systems and the evolution of color patterns: sensory processing illuminates signal evolution. *Evolution*, **59**, 1795–1818.
- Eriksson, J., Larson, G., Gunnarsson, U., Bed'hom, B., Tixier-Boichard, M., Strömstedt, L., Wright, D., Jungerius, A., Vereijken, A., Randi, E., Jensen, P. & Andersson, L.** 2008.

Identification of the yellow skin gene reveals a hybrid origin of the domestic chicken. *PLoS Genetics*, **4**, e1000010.

Falconer, D. S. & Mackay, T. F. C. 1996. *Introduction to quantitative genetics*. Essex: Longman.

Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J. & Cone, R. D. 1997. Role of melanocortineric neurons in feeding and the agouti obesity syndrome. *Nature*, **385**, 165–168.

Fang, M., Larson, G., Ribeiro, H. S., Li, N. & Andersson, L. 2009. Contrasting mode of evolution at a coat color locus in wild and domestic pigs. *PLoS Genetics*, **5**, e1000341.

Fisher, R. 1958. *The genetical theory of natural selection*. 2nd edn. New York: Dover.

Garant, D., Sheldon, B. C. & Gustafsson, L. 2004. Climatic and temporal effects on the expression of secondary sexual characters: genetic and environmental components. *Evolution*, **58**, 634–644.

Gasparini, J., Bize, P., Piault, R., Wakamatsu, K., Blount, J. D., Ducrest, A.-L. & Roulin, A. 2009. Strength and cost of an induced immune response are associated with a heritable melanin-based colour trait in female tawny owls. *The Journal of Animal Ecology*, **78**, 608–616.

Goldstein, G., Flory, K. R., Browne, B. A., Majid, S., Ichida, J. M. & Burt, E. H. 2004. Bacterial degradation of black and white feathers. *The Auk*, **121**, 656–659.

Gosler, a G., Barnett, P. R. & Reynolds, S. J. 2000. Inheritance and variation in eggshell patterning in the great tit *Parus major*. *Proceedings of the National Academy of Sciences*, **267**, 2469–2473.

Gosler, A. G., Higham, J. P. & James Reynolds, S. 2005. Why are birds' eggs speckled? *Ecology Letters*, **8**, 1105–1113.

Göth, A. & Evans, C. S. 2004. Social responses without early experience: Australian brush-turkey chicks use specific visual cues to aggregate with conspecifics. *Journal of Experimental Biology*, **207**, 2199–2208.

Grant, B. R. 1985. Selection on bill characters in a population of Darwin's finches: *Geospiza conirostris* on Isla Genoves, Galapagos. *Evolution*, **39**, 523–532.

Grant, B. R. 1990. The significance of subadult plumage in Darwin's finches, *Geospiza fortis*. *Behavioral Ecology*, **1**, 161–170.

Gratten, J., Beraldi, D., Lowder, B. V, McRae, a F., Visscher, P. M., Pemberton, J. M. & Slate, J. 2007. Compelling evidence that a single nucleotide substitution in TYRP1 is responsible for coat-colour polymorphism in a free-living population of Soay sheep. *Proceedings of the Royal Society B: Biological Sciences*, **274**, 619–626.

- Griffith, S. C., Parker, T. H. & Olson, V. A.** 2006. Melanin- versus carotenoid-based sexual signals: is the difference really so black and red? *Animal Behaviour*, **71**, 749–763.
- Groothuis, T. G. G. & von Engelhardt, N.** 2005. Investigating maternal hormones in avian eggs: measurement, manipulation, and interpretation. *Annals of the New York Academy of Sciences*, **1046**, 168–180.
- Gross, J. B., Borowsky, R. & Tabin, C. J.** 2009. A novel role for Mc1r in the parallel evolution of depigmentation in independent populations of the cavefish *Astyanax mexicanus*. *PLoS Genetics*, **5**, e1000326.
- Hadfield, J. D.** 2010. MCMC methods for multi-response generalized linear mixed models: The MCMCglmm R package. *Journal of Statistical Software*, **33**, 1–22.
- Hadfield, J. D. & Owens, I. P. F.** 2006. Strong environmental determination of a carotenoid-based plumage trait is not mediated by carotenoid availability. *Journal of Evolutionary Biology*, **19**, 1104–1114.
- Haldane, J. B. S.** 1930. A mathematical theory of artificial and natural selection. Part VI. Isolation. *Mathematical Proceedings of the Cambridge Philosophical Society*, **26**, 220–230.
- Hanotte, O., Zanon, C., Pugh, A., Greig, C., Dixon, A. & Burke, T.** 1994. Isolation and characterization of microsatellite loci in a passerine bird: the reed bunting *Emberiza schoeniclus*. *Molecular Ecology*, **3**, 529–530.
- Hansen, T. F. & Houle, D.** 2008. Measuring and comparing evolvability and constraint in multivariate characters. *Journal of Evolutionary Biology*, **21**, 1201–1219.
- Haywood, S. & Perrins, C. M.** 1992. Is clutch size in birds affected by environmental conditions during growth? *Proceedings of the Royal Society B: Biological Sciences*, **249**, 195–197.
- Heinsohn, R.** 2008. The ecological basis of unusual sex roles in reverse-dichromatic eclectus parrots. *Animal Behaviour*, **76**, 97–103.
- Heinsohn, R., Legge, S. & Endler, J. A.** 2005. Extreme reversed sexual dichromatism in a bird without sex role reversal. *Science*, **309**, 617–619.
- Hill, G. E.** 1991. Plumage coloration is a sexually selected indicator of male quality. *Nature*, **350**, 337–339.
- Hill, G. E.** 2006. Female mate choice for ornamental coloration. In: *Bird Coloration: Function and Evolution*, (Ed. by G. E. Hill & K. J. McGraw), pp. 137–200. Cambridge: Harvard University Press.

- Hine, E., Chenoweth, S. F., Rundle, H. D. & Blows, M. W.** 2009. Characterizing the evolution of genetic variance using genetic covariance tensors. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **364**, 1567–1578.
- Hine, E., McGuigan, K. & Blows, M. W.** 2011. Natural selection stops the evolution of male attractiveness. *Proceedings of the National Academy of Sciences*, **108**, 3659–3664.
- Hoekstra, H. E.** 2006. Genetics, development and evolution of adaptive pigmentation in vertebrates. *Heredity*, **97**, 222–234.
- Hoekstra, H. E. & Nachman, M. W.** 2003. Different genes underlie adaptive melanism in different populations of rock pocket mice. *Molecular Ecology*, **12**, 1185–1194.
- Hoekstra, H. E. & Price, T. D.** 2004. Parallel evolution is in the genes. *Science*, **303**, 1779–1780.
- Hoekstra, H. E., Drumm, K. E. & Nachman, M. W.** 2004. Ecological genetics of adaptive color polymorphism in pocket mice: geographic variation in selected and neutral genes. *Evolution*, **58**, 1329–1341.
- Hoekstra, H. E., Hirschmann, R. J., Bunday, R. A., Insel, P. A. & Crossland, J. P.** 2006. A single amino acid mutation contributes to adaptive beach mouse color pattern. *Science*, **313**, 101–104.
- Hohenlohe, P. A. & Arnold, S. J.** 2008. MIPoD: a hypothesis-testing framework for microevolutionary inference from patterns of divergence. *The American Naturalist*, **171**, 366–385.
- Hubbard, J. K., Uy, J. A. C., Hauber, M. E., Hoekstra, H. E. & Safran, R. J.** 2010. Vertebrate pigmentation: from underlying genes to adaptive function. *Trends in Genetics*, **26**, 231–239.
- Hughes, K. A., Rodd, F. H. & Reznick, D. N.** 2005. Genetic and environmental effects on secondary sex traits in guppies (*Poecilia reticulata*). *Journal of Evolutionary Biology*, **18**, 35–45.
- Hunt, J., Wolf, J. B. & Moore, A. J.** 2007. The biology of multivariate evolution. *Journal of Evolutionary Biology*, **20**, 24–27.
- Iljin, N. & Iljin, V.** 1930. Temperature effects on the color of the Siamese cat. *Journal of Heredity*, **21**, 309–318.
- Ingleby, F. C., Hunt, J. & Hosken, D. J.** 2010. The role of genotype-by-environment interactions in sexual selection. *Journal of Evolutionary Biology*, **23**, 2031–2045.
- Iwasa, Y. & Pomiankowski, A.** 1999. Good parent and good genes models of handicap evolution. *Journal of Theoretical Biology*, **200**, 97–109.

- Jackson, I. J.** 1997. Homologous pigmentation mutations in human, mouse and other model organisms. *Human Molecular Genetics*, **6**, 1613–1624.
- Jackson, P. J., Douglas, N. R., Chai, B., Binkley, J., Sidow, A., Barsh, G. S. & Millhauser, G. L.** 2006. Structural and molecular evolutionary analysis of Agouti and Agouti-related proteins. *Chemistry & Biology*, **13**, 1297–1305.
- Jacquin, L., Lenouvel, P., Haussy, C., Ducatez, S. & Gasparini, J.** 2011. Melanin-based coloration is related to parasite intensity and cellular immune response in an urban free living bird: the feral pigeon *Columba livia*. *Journal of Avian Biology*, **42**, 11–15.
- Jenkins, B. R., Vitousek, M. N. & Safran, R. J.** 2013. Signaling stress? An analysis of pheomelanin-based plumage color and individual corticosterone levels at two temporal scales in North American barn swallows, *Hirundo rustica erythrogaster*. *Hormones and Behavior*, **64**, 665–672.
- Jenkins, B. R., Vitousek, M. N., Hubbard, J. K. & Safran, R. J.** 2014. An experimental analysis of the heritability of variation in glucocorticoid concentrations in a wild avian population. *Proceedings of the Royal Society B: Biological Sciences*, **281**, 20141302.
- Jensen, J. D., Wong, A. & Aquadro, C. F.** 2007. Approaches for identifying targets of positive selection. *Trends in Genetics*, **23**, 568–577.
- Johansson, F., Lind, M. I., Ingvarsson, P. K. & Bokma, F.** 2012. Evolution of the G-matrix in life history traits in the common frog during a recent colonisation of an island system. *Evolutionary Ecology*, **26**, 863–878.
- Johnsen, A., Delhey, K., Andersson, S. & Kempenaers, B.** 2003. Plumage colour in nestling blue tits: sexual dichromatism, condition dependence and genetic effects. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 1263–1270.
- Jones, A. G., Arnold, S. J. & Bürger, R.** 2003. Stability of the G-matrix in a population experiencing pleiotropic mutation, stabilizing selection, and genetic drift. *Evolution*, **57**, 1747–60.
- Kalinowski, S. T., Taper, M. L. & Marshall, T. C.** 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*, **16**, 1099–1106.
- Kelsh, R. N.** 2004. Genetics and Evolution of Pigment Patterns in Fish. *Pigment Cell Research*, **17**, 326–336.
- Kim, Y. & Stephan, W.** 2002. Detecting a local signature of genetic hitchhiking along a recombining chromosome. *Genetics*, **160**, 765–777.

- Kingsley, E. P., Manceau, M., Wiley, C. D. & Hoekstra, H. E.** 2009. Melanism in peromyscus is caused by independent mutations in agouti. *PLoS One*, **4**, e6435.
- Kirkpatrick, M.** 1996. Good genes and direct selection in the evolution of mating preferences. *Evolution*, **50**, 2125–2140.
- Kleven, O., Jacobsen, F., Izadnegahdar, R., Robertson, R. J. & Lifjeld, J. T.** 2006. Male tail streamer length predicts fertilization success in the North American barn swallow (*Hirundo rustica erythrogaster*). *Behavioral Ecology and Sociobiology*, **59**, 412–418.
- Klovins, J. & Schiöth, H. B.** 2005. Agouti-related proteins (AGRP) and agouti-signaling peptide (ASIP) in fish and chicken. *Annals of the New York Academy of Sciences*, **1040**, 363–367.
- Kokko, H.** 1998. Should advertising parental care be honest? *Proceedings of the Royal Society B: Biological Sciences*, **265**, 1871–1878.
- Kokko, H. & Johnstone, R. a.** 2002. Why is mutual mate choice not the norm? Operational sex ratios, sex roles and the evolution of sexually dimorphic and monomorphic signalling. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **357**, 319–330.
- Kokko, H., Brooks, R., Jennions, M. D. & Morley, J.** 2003. The evolution of mate choice and mating biases. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 653–664.
- Kruuk, L. E. B.** 2004. Estimating genetic parameters in natural populations using the “animal model”. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **359**, 873–890.
- Kruuk, L. E. B. & Hadfield, J. D.** 2007. How to separate genetic and environmental causes of similarity between relatives. *Journal of Evolutionary Biology*, **20**, 1890–1903.
- Kwon, B. S., Haq, A. K., Wakulchik, M., Kestler, D., Barton, D. E., Francke, U., Lamoreux, M. L., Whitney III, J. B. & Halaban, R.** 1989. Isolation, chromosomal mapping, and expression of the mouse tyrosinase gene. *Journal of Investigative Dermatology*, **93**,
- Lalueza-Fox, C., Römpler, H., Caramelli, D., Stäubert, C., Catalano, G., Hughes, D., Rohland, N., Pilli, E., Longo, L., Condemi, S., de La Rasilla, M., Fortea, J., Rosas, A., Stoneking, M., Schöneberg, T., Bertranpetit, J. & Hofreiter, M.** 2007. A melanocortin 1 receptor allele suggests varying pigmentation among Neanderthals. *Science*, **318**, 1453–1455.
- Lamason, R. L., Mohideen, M. P. K., Mest, J. R., Wong, A. C., Norton, H. L., Aros, M. C., Juryne, M. J., Mao, X., Humphreys, V. R., Humbert, J. E., Sinha, S., Moore, J. L., Jagadeeswaran, P., Zhao, W., Ning, G., Makalowska, I., Mckeigue, P. M., Donnell, D. O., Kittles, R., Parra, E. J., Mangini, N. J., Grunwald, D. J., Shriver, M. D., Canfield, V. A. & Cheng, K. C.** 2005. SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. *Science*, **310**, 1782–1786.

- Lande, R.** 1979. Quantitative genetic analysis of multivariate evolution, applied to brain: body size allometry. *Evolution*, **33**, 402–416.
- Lande, R.** 1980. The genetic covariance between characters maintained by pleiotropic mutations. *Genetics*, **94**, 203–215.
- Lindsay, W. R., Webster, M. S. & Schwabl, H.** 2011. Sexually Selected Male Plumage Color Is Testosterone Dependent in a Tropical Passerine Bird, the Red-Backed Fairy-Wren (*Malurus melanocephalus*). *PLoS One*, **6**, e26067.
- Lindström, J.** 1999. Early development and fitness in birds and mammals. *Trends in Ecology & Evolution*, **14**, 343–348.
- Linnen, C. R., Kingsley, E. P., Jensen, J. D. & Hoekstra, H. E.** 2009. On the origin and spread of an adaptive allele in deer mice. *Science*, **325**, 1095–1098.
- Ludwig, A., Pruvost, M., Reissmann, M., Benecke, N., Brockmann, G. A., Castaños, P., Cieslak, M., Lippold, S., Llorente, L., Malaspinas, A.-S., Slatkin, M. & Hofreiter, M.** 2009. Coat color variation at the beginning of horse domestication. *Science*, **324**, 485.
- Lynch, M. & Walsh, B.** 1998. *Genetics and analysis of quantitative traits*. Sunderland: Sinauer.
- MacDonald, I. F., Kempster, B., Zanette, L. & MacDougall-Shackleton, S. A.** 2006. Early nutritional stress impairs development of a song-control brain region in both male and female juvenile song sparrows (*Melospiza melodia*) at the onset of song learning. *Proceedings of the Royal Society B: Biological Sciences*, **273**, 2559–2564.
- Maia, R., Eliason, C., Bitton, P.-P., Doucet, S. M. & Shawkey, M. D.** 2013. pavo: an R package for the analysis, visualization and organization of spectral data. *Methods in Ecology and Evolution*
- Mancuso, K., Hauswirth, W. W., Li, Q., Connor, T. B., Kuchenbecker, J. a, Mauck, M. C., Neitz, J. & Neitz, M.** 2009. Gene therapy for red-green colour blindness in adult primates. *Nature*, **461**, 784–787.
- McDonald, D. B. & Potts, W. K.** 1994. Cooperative display and relatedness among males in a lek-mating bird. *Science*, **266**, 1030–1032.
- McGraw, K. J.** 2006a. Mechanics of melanin-based coloration. In: *Bird Coloration: Mechanisms and Measurement*, (Ed. by G. E. Hill & K. J. McGraw), pp. 243–294. Cambridge: Harvard University Press.
- McGraw, K. J.** 2006b. Mechanisms of carotenoid-based coloration. In: *Bird Coloration: Mechanisms and Measurement*, (Ed. by G. E. Hill & K. J. McGraw), Cambridge: Harvard University Press.

- McGraw, K. J.** 2006c. Mechanics of uncommon colors: pterines, porphyrins, and psittacofulvins. In: *Bird Coloration: Mechanisms and Measurement*, (Ed. by G. E. Hill & K. J. McGraw), pp. 354–398. Cambridge: Harvard University Press.
- McGraw, K. J.** 2006d. Dietary mineral content influences the expression of melanin-based ornamental coloration. *Behavioral Ecology*, **18**, 137–142.
- McGraw, K. J.** 2008. An update on the honesty of melanin-based color signals in birds. *Pigment cell & melanoma research*, **21**, 133–138.
- McGraw, K. J., Safran, R. J., Evans, M. R. & Wakamatsu, K.** 2004. European barn swallows use melanin pigments to color their feathers brown. *Behavioral Ecology*, **15**, 889–891.
- McGraw, K. J., Safran, R. J. & Wakamatsu, K.** 2005. How feather colour reflects its melanin content. *Functional Ecology*, **19**, 816–821.
- McKinnon, J. S. & Pierotti, M. E. R.** 2010. Colour polymorphism and correlated characters: genetic mechanisms and evolution. *Molecular Ecology*, **19**, 5101–5125.
- Meiklejohn, C. D., Kim, Y., Hartl, D. L. & Parsch, J.** 2004. Identification of a locus under complex positive selection in *Drosophila simulans* by haplotype mapping and composite-likelihood estimation. *Genetics*, **168**, 265–279.
- Merilä, J. & Svensson, E.** 1997. Are fat reserves in migratory birds affected by condition in early life? *Journal of Avian Biology*, **28**, 279–286.
- Miller, C. T., Beleza, S., Pollen, A. A., Schluter, D., Kittles, R. A., Shriver, M. D. & Kingsley, D. M.** 2007. cis-Regulatory changes in Kit ligand expression and parallel evolution of pigmentation in sticklebacks and humans. *Cell*, **131**, 1179–1189.
- Mills, M. G. & Patterson, L. B.** 2009. Not just black and white: pigment pattern development and evolution in vertebrates. *Seminars in Cell & Developmental Biology*, **20**, 72–81.
- Minvielle, F., Gourichon, D., Ito, S., Inoue-Murayama, M. & Rivière, S.** 2007. Effects of the dominant lethal yellow mutation on reproduction, growth, feed consumption, body temperature, and body composition of the Japanese quail. *Poultry Science*, **86**, 1646–1650.
- Moczek, A. P. & Emlen, D. J.** 1999. Proximate determination of male horn dimorphism in the beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *Journal of Evolutionary Biology*, **12**, 27–37.
- Møller, A. P.** 1994. *Sexual selection and the barn swallow*. New York: Oxford University Press.
- Møller, A. P., Barbosa, A., Cuervo, J. J., de Lope, F., Merino, S. & Saino, N.** 1998. Sexual selection and tail streamers in the barn swallow. *Proceedings of the Royal Society B: Biological Sciences*, **265**, 409–414.

- Montgomerie, R.** 2006. Analyzing colors. In: *Bird Coloration: Mechanisms and Measurement*, (Ed. by G. E. Hill & K. J. McGraw), pp. 90–147. Cambridge: Harvard University Press.
- Mountjoy, K. G., Robbins, L. S., Mortrud, M. T. & Cone, R. D.** 1992. The cloning of a family of genes that encode the melanocortin receptors. *Science*, **257**, 1248–1251.
- Mousseau, T. A. & Fox, C. W.** 1998. The adaptive significance of of maternal effects. *Trends in Ecology & Evolution*, **13**, 403–407.
- Mullen, L. M., Vignieri, S. N., Gore, J. A. & Hoekstra, H. E.** 2009. Adaptive basis of geographic variation: genetic, phenotypic and environmental differences among beach mouse populations. *Proceedings of the Royal Society B: Biological Sciences*, **276**, 3809–3818.
- Müller, W., Goerlich, V. C., Vergauwen, J., Groothuis, T. G. G. & Eens, M.** 2012. Sources of variation in yolk hormone deposition: consistency, inheritance and developmental effects. *General and comparative endocrinology*, **175**, 337–343.
- Mundy, N. I.** 2005. A window on the genetics of evolution: MC1R and plumage colouration in birds. *Proceedings of the Royal Society B: Biological Sciences*, **272**, 1633–1640.
- Mundy, N. I.** 2006. Genetic basis of color variation in wild birds. In: *Bird Coloration: Mechanisms and Measurement*, (Ed. by G. E. Hill & K. J. McGraw), pp. 469–506. Cambridge: Harvard University Press.
- Mundy, N. I. & Kelly, J.** 2006. Investigation of the role of the agouti signaling protein gene (ASIP) in coat color evolution in primates. *Mammalian Genome*, **17**, 1205–1213.
- Mundy, N. I., Badcock, N. S., Hart, T., Scribner, K., Janssen, K. & Nadeau, N. J.** 2004. Conserved genetic basis of a quantitative plumage trait involved in mate choice. *Science*, **303**, 1870–1873.
- Nachman, M. W., Hoekstra, H. E. & D’Agostino, S. L.** 2003. The genetic basis of adaptive melanism in pocket mice. *Proceedings of the National Academy of Sciences*, **100**, 5268–5273.
- Nadeau, N. J., Mundy, N. I., Gourichon, D. & Minvielle, F.** 2007. Association of a single-nucleotide substitution in TYRP1 with roux in Japanese quail (*Coturnix japonica*). *Animal Genetics*, **38**, 609–613.
- Nadeau, N. J., Minvielle, F., Ito, S., Inoue-Murayama, M., Gourichon, D., Follett, S. A., Burke, T. & Mundy, N. I.** 2008. Characterization of Japanese quail yellow as a genomic deletion upstream of the avian homolog of the mammalian ASIP (agouti) gene. *Genetics*, **178**, 777–786.
- Neuman, C. R., Safran, R. J. & Lovette, I. J.** 2007. Male tail streamer length does not predict apparent or genetic reproductive success in North American barn swallows *Hirundo rustica erythrogaster*. *Journal of Avian Biology*, **38**, 28–36.

- Nielsen, R.** 2005. Molecular signatures of natural selection. *Annual Review of Genetics*, **39**, 197–218.
- Nowicki, S., Peters, S. & Podos, J.** 1998. Song learning, early nutrition and sexual selection in songbirds. *American Zoology*, **38**, 179–190.
- Nowicki, S., Searcy, W. A. & Peters, S.** 2002. Brain development, song learning and mate choice in birds: a review and experimental test of the “nutritional stress hypothesis”. *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology*, **188**, 1003–1014.
- Ödeen, A., Hart, N. S. & Håstad, O.** 2009. Assessing the use of genomic DNA as a predictor of the maximum absorbance wavelength of avian SWS1 opsin visual pigments. *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology*, **195**, 8308–8313.
- Ohlsson, T., Smith, H. G., Råberg, L. & Hasselquist, D.** 2002. Pheasant sexual ornaments reflect nutritional conditions during early growth. *Proceedings of the Royal Society B: Biological Sciences*, **269**, 21–27.
- Ollmann, M. M., Lamoreux, M. L., Wilson, B. D. & Barsh, G. S.** 1998. Interaction of Agouti protein with the melanocortin 1 receptor in vitro and in vivo. *Genes & Development*, **12**, 316–330.
- Osorio, D. & Vorobyev, M.** 2008. A review of the evolution of animal colour vision and visual communication signals. *Vision Research*, **48**, 2042–2051.
- Owen, J. P., Nelson, A. C. & Clayton, D. H.** 2010. Ecological immunology of bird-ectoparasite systems. *Trends in Parasitology*, **26**, 530–539.
- Phillips, P. C. & Arnold, S. J.** 1989. Visualizing multivariate selection. *Evolution*, **43**, 1209–1222.
- Phillips, P. C., Whitlock, M. C. & Fowler, K.** 2001. Inbreeding changes the shape of the genetic covariance matrix in *Drosophila melanogaster*. *Genetics*, **158**, 1137–1145.
- Potti, J. & Montalvo, S.** 2008. Male colour variation in Spanish Pied Flycatchers *Ficedula hypoleuca*. *Ibis*, **133**, 293–299.
- Primmer, C. R., Møller, A. P. & Ellegren, H.** 1995. Resolving genetic relationships with microsatellite markers: a parentage testing system for the swallow *Hirundo rustica*. *Molecular Ecology*, **4**, 493–498.
- Protas, M. E. & Patel, N. H.** 2008. Evolution of coloration patterns. *Annual Review of Cell and Developmental Biology*, **24**, 425–446.

- Protas, M. E., Hersey, C., Kochanek, D., Zhou, Y., Wilkens, H., Jeffery, W. R., Zon, L. I., Borowsky, R. & Tabin, C. J.** 2006. Genetic analysis of cavefish reveals molecular convergence in the evolution of albinism. *Nature Genetics*, **38**, 107–111.
- Prum, R. O.** 2006. Anatomy, physics, and evolution of structural colors. In: *Bird Coloration: Mechanisms and Measurement*, (Ed. by G. E. Hill & K. J. McGraw), Harvard University Press.
- Prum, R. O., Dufresne, E. R., Quinn, T. & Waters, K.** 2009. Development of colour-producing beta-keratin nanostructures in avian feather barbs. *Journal of the Royal Society, Interface*, **6 Suppl 2**, S253–S265.
- Punzalan, D., Cooray, M., Helen Rodd, F. & Rowe, L.** 2008. Condition dependence of sexually dimorphic colouration and longevity in the ambush bug *Phymata americana*. *Journal of Evolutionary Biology*, **21**, 1297–1306.
- Quesada, J. & Senar, J. C.** 2009. Cross-fostering experiments to compare carotenoid- and melanin-based plumage traits and long-term parental effects in post-moulted great tits. *Behaviour*, **146**, 1235–1251.
- Räsänen, K. & Kruuk, L. E. B.** 2007. Maternal effects and evolution at ecological time-scales. *Functional Ecology*, **21**, 408–421.
- Ritland, K., Newton, C. & Marshall, H. D.** 2001. Inheritance and population structure of the white-phased. *Current Biology*, **11**, 1468–1472.
- Roberts, R. B., Ser, J. R. & Kocher, T. D.** 2009. Sexual conflict resolved by invasion of a novel sex determiner in Lake Malawi cichlid fishes. *Science*, **326**, 998–1001.
- Roff, D. a & Fairbairn, D. J.** 2012. The evolution of trade-offs under directional and correlational selection. *Evolution*, **66**, 2461–2474.
- Römpler, H., Rohland, N., Lalueza-Fox, C., Willerslev, E., Kuznetsova, T., Rabeder, G., Bertranpetit, J., Schöneberg, T. & Hofreiter, M.** 2006. Nuclear gene indicates coat-color polymorphism in mammoths. *Science*, **313**, 62.
- Rooney, K. & Ozanne, S. E.** 2011. Maternal over-nutrition and offspring obesity predisposition: targets for preventative interventions. *International Journal of Obesity*, **35**, 883–890.
- Rosenblum, E. B., Hoekstra, H. E. & Nachman, M. W.** 2004. Adaptive reptile color variation and the evolution of the Mc1r gene. *Evolution*, **58**, 1794–1808.
- Rosenblum, E. B., Römpler, H., Schöneberg, T. & Hoekstra, H. E.** 2010. Molecular and functional basis of phenotypic convergence in white lizards at White Sands. *Proceedings of the National Academy of Sciences*, **107**, 2113–2117.

- Roulin, A.** 2004. The evolution, maintenance and adaptive function of genetic colour polymorphism in birds. *Biological reviews of the Cambridge Philosophical Society*, **79**, 815–848.
- Roulin, A. & Dijkstra, C.** 2003. Genetic and environmental components of variation in eumelanin and pheomelanin sex-traits in the barn owl. *Heredity*, **90**, 359–364.
- Rubenstein, D. R. & Hauber, M. E.** 2008. Dynamic feedback between phenotype and physiology in sexually selected traits. *Trends in Ecology & Evolution*, **23**, 655–658.
- Safran, R. J.** 2007. Settlement patterns of female barn swallows *Hirundo rustica* across different group sizes: access to colorful males or favored nests? *Behavioral Ecology and Sociobiology*, **61**, 1359–1368.
- Safran, R. J. & McGraw, K. J.** 2004. Plumage coloration, not length or symmetry of tail-streamers, is a sexually selected trait in North American barn swallows. *Behavioral Ecology*, **15**, 455–461.
- Safran, R. J., Neuman, C. R., McGraw, K. J. & Lovette, I. J.** 2005. Dynamic paternity allocation as a function of male plumage color in barn swallows. *Science*, **309**, 2210–2212.
- Safran, R. J., Adelman, J. S., McGraw, K. J. & Hau, M.** 2008a. Sexual signal exaggeration affects physiological state in male barn swallows. *Current Biology*, **18**, 461–462.
- Safran, R. J., Pilz, K. M., McGraw, K. J., Correa, S. M. & Schwabl, H.** 2008b. Are yolk androgens and carotenoids in barn swallow eggs related to parental quality? *Behavioral Ecology and Sociobiology*, **62**, 427–438.
- Safran, R. J., McGraw, K. J., Wilkins, M. R., Hubbard, J. K. & Marling, J.** 2010a. Positive carotenoid balance correlates with greater reproductive performance in a wild bird. *PLoS One*, **5**, e9420.
- Safran, R. J., McGraw, K. J., Pilz, K. M. & Correa, S. M.** 2010b. Egg-yolk androgen and carotenoid deposition as a function of maternal social environment in barn swallows *Hirundo rustica*. *Journal of Avian Biology*, **41**, 470–478.
- Safran, R. J., Scordato, E. S. C., Symes, L. B., Rodríguez, R. L. & Mendelson, T. C.** 2013. Contributions of natural and sexual selection to the evolution of premating reproductive isolation: a research agenda. *Trends in Ecology & Evolution*, **28**, 643–650.
- Saino, N., Primmer, C. R., Ellegren, H. & Møller, A. P.** 1997. An experimental study of paternity and tail ornamentation in the barn swallow (*Hirundo rustica*). *Evolution*, **51**, 562–570.
- Saino, N., Romano, M., Rubolini, D., Teplitsky, C., Ambrosini, R., Caprioli, M., Canova, L. & Wakamatsu, K.** 2013. Sexual dimorphism in melanin pigmentation, feather coloration and its heritability in the barn swallow (*Hirundo rustica*). *PLoS One*, **8**, e58024.

- Schmidt-Küntzel, A., Eizirik, E., O'Brien, S. J. & Menotti-Raymond, M.** 2005. Tyrosinase and tyrosinase related protein 1 alleles specify domestic cat coat color phenotypes of the albino and brown loci. *The Journal of Heredity*, **96**, 289–301.
- Scordato, E. S. C., Bontrager, A. L. & Price, T. D.** 2012. Cross-generational effects of climate change on expression of a sexually selected trait. *Current Biology*, **22**, 78–82.
- Seehausen, O., Terai, Y., Magalhaes, I. S., Carleton, K. L., Mrosso, H. D. J., Miyagi, R., van der Sluijs, I., Schneider, M. V, Maan, M. E., Tachida, H., Imai, H. & Okada, N.** 2008. Speciation through sensory drive in cichlid fish. *Nature*, **455**, 620–626.
- Senar, J. C.** 2006. Color displays as intrasexual signals of aggression and dominance. In: *Bird Coloration: Function and Evolution*, (Ed. by G. E. Hill & K. J. McGraw), pp. 87–136. Cambridge: Harvard University Press.
- Servedio, M. R. & Lande, R.** 2006. Population genetic models of male and mutual mate choice. *Evolution*, **60**, 674–685.
- Shi, Y. & Yokoyama, S.** 2003. Molecular analysis of the evolutionary significance of ultraviolet vision in vertebrates. *Proceedings of the National Academy of Sciences*, **100**, 8308–8313.
- Siefferman, L. & Hill, G. E.** 2005. Evidence for sexual selection on structural plumage coloration in female eastern bluebirds (*Sialia sialis*). *Evolution*, **59**, 1819–1828.
- Silvers, W. K.** 1979. *The coat colors of mice: a model for mammalian gene action and interaction*. New York: Springer-Verlag.
- Slagsvold, T., Dale, S. & Kruszewicz, A.** 1995. Predation favours cryptic coloration in breeding male pied flycatchers. *Animal Behaviour*, **50**, 1109–1121.
- Slagsvold, T., Hansen, B. T., Johannessen, L. E. & Lifjeld, J. T.** 2002. Mate choice and imprinting in birds studied by cross-fostering in the wild. *Proceedings of the Royal Society B: Biological Sciences*, **269**, 1449–1455.
- Spencer, K. A., Buchanan, K. L., Goldsmith, A. R. & Catchpole, C. K.** 2003. Song as an honest signal of developmental stress in the zebra finch (*Taeniopygia guttata*). *Hormones and Behavior*, **44**, 132–139.
- Steffen, J. E. & McGraw, K. J.** 2007. Contributions of pterin and carotenoid pigments to dewlap coloration in two anole species. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, **146**, 42–46.
- Steiner, C. C., Weber, J. N. & Hoekstra, H. E.** 2007. Adaptive variation in beach mice produced by two interacting pigmentation genes. *PLoS Biology*, **5**, e219.

- Steiner, C. C., Römpler, H., Boettger, L. M., Schöneberg, T. & Hoekstra, H. E.** 2009. The genetic basis of phenotypic convergence in beach mice: similar pigment patterns but different genes. *Molecular Biology and Evolution*, **26**, 35–45.
- Stoddard, M. C. & Prum, R. O.** 2008. Evolution of avian plumage color in a tetrahedral color space: a phylogenetic analysis of new world buntings. *The American Naturalist*, **171**, 755–776.
- Sturm, R. a.** 2009. Molecular genetics of human pigmentation diversity. *Human Molecular Genetics*, **18**, R9–R17.
- Sturm, R. a & Frudakis, T. N.** 2004. Eye colour: portals into pigmentation genes and ancestry. *Trends in Genetics*, **20**, 327–332.
- Team, R. C.** 2014. R: A language and environment for statistical computing.
- Theron, E., Hawkins, K., Bermingham, E., Ricklefs, R. E. & Mundy, N. I.** 2001. The molecular basis of an avian plumage polymorphism in the wild: a melanocortin-1-receptor point mutation is perfectly associated with the melanic plumage morph of the bananaquit, *Coereba flaveola*. *Current Biology*, **11**, 550–557.
- Tilgar, V., Mänd, R., Kilgas, P. & Mägi, M.** 2009. Long-term consequences of early ontogeny in free-living Great Tits *Parus major*. *Journal of Ornithology*, **151**, 61–68.
- Tschirren, B., Postma, E., Rutstein, A. N. & Griffith, S. C.** 2012. When mothers make sons sexy: maternal effects contribute to the increased sexual attractiveness of extra-pair offspring. *Proceedings of the Royal Society B: Biological Sciences*, **279**, 1233–1240.
- Tsyusko, O. V., Peters, M. B., Hagen, C., Tuberville, T. D., Mousseau, T. a., Møller, A. P. & Glenn, T. C.** 2007. Microsatellite markers isolated from barn swallows (*Hirundo rustica*). *Molecular Ecology Notes*, **7**, 833–835.
- Turner, A.** 2006. *The Barn Swallow*. London: T & A D Poyser.
- Uy, J. A. C., Moyle, R. G., Filardi, C. E. & Cheviron, Z. A.** 2009. Difference in plumage color used in species recognition between incipient species is linked to a single amino acid substitution in the melanocortin-1 receptor. *The American Naturalist*, **174**, 244–254.
- Våge, D. I., Lu, D., Klungland, H., Lien, S., Adalsteinsson, S. & Cone, R. D.** 1997. A non-epistatic interaction of agouti and extension in the fox, *Vulpes vulpes*. *Nature Genetics*, **15**, 311–315.
- Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M. & Shipley, P.** 2004. Micro-Checker: Software for Identifying and Correcting Genotyping Errors in Microsatellite Data. *Molecular Ecology Notes*, **4**, 535–538.

- Verhulst, S, Perrins, C. M. & Riddington, R.** 1997. Natal Dispersal of Great Tits in a Patchy Environment. *Ecology*, **78**, 864–872.
- Vitousek, M. N., Stewart, R. A. & Safran, R. J.** 2013. Female plumage colour influences seasonal oxidative damage and testosterone profiles in a songbird. *Biology Letters*, **9**, 20130539.
- Vortman, Y., Lotem, A., Dor, R., Lovette, I. J. & Safran, R. J.** 2011. The sexual signals of the East-Mediterranean barn swallow: a different swallow tale. *Behavioral Ecology*, **22**, 1344–1352.
- Vortman, Y., Lotem, A., Dor, R., Lovette, I. & Safran, R. J.** 2013. Multiple sexual signals and behavioral reproductive isolation in a diverging population. *The American Naturalist*, **182**, 514–523.
- Walsh, B.** 2014. Special issues on advances in quantitative genetics: introduction. *Heredity*, **112**, 1–3.
- Wehrle-Haller, B.** 2003. The role of Kit-ligand in melanocyte development and epidermal homeostasis. *Pigment Cell Research*, **16**, 287–296.
- White, J. M., Legates, J. E. & Eisen, E. J.** 1968. Maternal effects among lines of mice selected for body weight. *Genetics*, **60**, 395–408.
- Willier, B. & Rawles, M. E.** 1940. The control of feather color pattern by melanophores grafted from one embryo to another of a different breed of fowl. *Physiological Zoology*, **13**, 177–201.
- Wilson, A. J., Réale, D., Clements, M. N., Morrissey, M. M., Postma, E., Walling, C. A., Kruuk, L. E. B. & Nussey, D. H.** 2010. An ecologist's guide to the animal model. *The Journal of Animal Ecology*, **79**, 13–26.
- Wolak, M., Fairbairn, D. J. & Paulsen, Y. R.** 2012. Guidelines for estimating repeatability. *Methods in Ecology and Evolution*, **3**, 129–137.
- Zdarsky, E., Favor, J. & Jackson, I. J.** 1990. The molecular basis of brown, an old mouse mutation, and of an induced revertant to wild type. *Genetics*, **126**, 443–449.

8. Appendix

Table 1. Pigmentation genes identified to affect on coloration in wild populations: polymorphisms and their phenotypic associations with melanin coloration in skin, scales, feathers, and fur

GENE	MUTATION	SPECIES	CLASS	DERIVED PHENOTYPE	REF
<i>MC1R</i>	Arg164Cys	<i>Asyanax mexicanus</i>	Actinopterygii	Darker skin	[1]
	MC1R-Δ2 - 2 bp deletion	<i>Asyanax mexicanus</i>	Actinopterygii	Darker skin	[1]
	Val85Met	<i>Anser c. caerulescens</i>	Aves	Darker plumage	[2]
	3' noncoding 960	<i>Coereba flaveola</i>	Aves	Darker plumage	[3]
	Glu92Lys ^{A,B}	<i>Coereba flaveola</i>	Aves	Darker plumage	[3]
	Glu92Lys	<i>Coturnix japonica</i>	Aves	Darker plumage	[4]
	Glu92Lys	<i>Gallus gallus</i>	Aves	Darker plumage	[5,6]
	Ala16Thr	<i>Malurus leucopterus</i>	Aves	Darker plumage	[7]
	Ile38Asp	<i>Malurus leucopterus</i>	Aves	Darker plumage	[7]
	Val11Ile	<i>Malurus leucopterus</i>	Aves	Darker plumage	[7]
	Glu157Arg	<i>Malurus leucopterus</i>	Aves	Darker plumage	[7]
	Val166Ile	<i>Malurus leucopterus</i>	Aves	Darker plumage	[7]
		<i>Monarcha castaneiventris</i>	Aves	Darker plumage	[8]
	Asp119Asn	<i>Stercorarius parasiticus</i>	Aves	Darker plumage	[2]
	Arg230His		Aves	Darker plumage	[9]
	Val85Met	<i>Sula sula</i>	Aves	Darker plumage	[9]
	His207Arg	<i>Sula sula</i>	Aves	Darker plumage	[9]
	Gly5Cys	<i>Alopex lagopus</i>	Mammalia	Darker coat	[10]
	Phe280Cys	<i>Alopex lagopus</i>	Mammalia	Darker coat	[10]
	Leu99Pro ^A	<i>Bos taurus</i>	Mammalia	Darker coat	[11]
	Arg109Trp ^C	<i>Chaetodipus intermedius</i>	Mammalia	Darker coat	[12,13]
	Arg160Trp ^{B,C}	<i>Chaetodipus intermedius</i>	Mammalia	Darker coat	[12,13]
	Arg18Cys ^C	<i>Chaetodipus intermedius</i>	Mammalia	Darker coat	[12,13]
	Gln233His ^C	<i>Chaetodipus intermedius</i>	Mammalia	Darker coat	[12,13]
	Ser83Phe	<i>Equus caballus</i>	Mammalia	Chestnut coat	[14]
	MC1R-Δ24 - 24 bp deletion	<i>Herpailurus yagouaroni</i>	Mammalia	Darker coat	[15]
	Arg307Gly	<i>Homo neanderthalensis</i>	Mammalia	Lighter skin?	[16]
	Arg67Cys	<i>Mammuthus primigenius</i>	Mammalia	Darker coat?	[17]

GENE	MUTATION	SPECIES	CLASS	DERIVED PHENOTYPE	REF	
MC1R	Asp119Asn ^C	<i>Ovis aries</i>	Mammalia	Darker coat	[18]	
	Met73Lys ^C	<i>Ovis aries</i>	Mammalia	Darker coat	[18]	
	MC1R-Δ15 - 15 bp deletion	<i>Panthera onca</i>	Mammalia	Darker coat	[15]	
	Arg65Cys	<i>Peromyscus polionotus</i>	Mammalia	Lighter coat	[19]	
	Asp121Asn ^C	<i>Sus scrofa</i>	Mammalia	Darker coat	[20]	
	Leu99Pro ^{A,C}	<i>Sus scrofa</i>	Mammalia	Darker coat	[20]	
	Tyr298Cys	<i>Ursus americanus</i>	Mammalia	Lighter coat	[21]	
	Cys125Arg	<i>Vulpes vulpes</i>	Mammalia	Darker coat	[22]	
	Thr170Ile	<i>Aspidoscelis inornata</i>	Reptilia	Lighter scales	[23,24]	
	Val168Ile	<i>Holbrookia maculata</i>	Reptilia	Lighter scales	[23,24]	
	His208Tyr	<i>Sceloporus undulatus</i>	Reptilia	Lighter scales	[23,24]	
	ASIP	ASIP-Δ8 - 8 bp deletion	<i>Coturnix japonica</i>	Aves	Lighter plumage	[25]
		Arg96Cys	<i>Canis lupus familiaris</i>	Mammalia	Darker coat	[26]
ASIP-Δ11 - 11 bp deletion in exon 2		<i>Equus caballus</i>	Mammalia	Darker coat	[14]	
ASIP-Δ2 - 2 bp deletion in exon 2		<i>Felis catus</i>	Mammalia	Darker coat	[15]	
Unknown non-coding mutation		<i>Ovis aries</i>	Mammalia	Darker coat	[27]	
Exon 2 deletion		<i>Peromyscus maniculatus</i>	Mammalia	Darker coat	[28]	
Premature stop codon = no exon 4		<i>Peromyscus maniculatus</i>	Mammalia	Darker coat	[28]	
cis-regulatory change		Peromyscus maniculatus	Mammalia	Lighter coat	[29]	
Ser deletion in exon 2		Peromyscus maniculatus	Mammalia	Lighter coat	[29]	
ASIP-Δ19 - 19 bp deletion in exon 2		<i>Rattus</i>	Mammalia	Darker coat	[30]	
ASIP-Δ166 - 166 bp deletion in exon 2		<i>Vulpes vulpes</i>	Mammalia	Darker coat	[22]	
TYRPI	Phe282Ser	<i>Coturnix japonica</i>	Aves	Lighter plumage	[31]	
	His434Tyr	<i>Bos taurus</i>	Mammalia	Lighter coat	[32]	
	Cys41Ser	<i>Canis lupus familiaris</i>	Mammalia	Lighter coat	[33]	
	Ala3Gly	<i>Felis catus</i>	Mammalia	Lighter coat	[34]	
	Cys290Phe	<i>Ovis aries</i>	Mammalia	Lighter coat	[35]	
OCA2	2 aa changes, deletion in intron 23 / exon 24	Asyanax mexicanus	Actinopterygii	Lighter skin	[36]	
	Exon 21 missing	Asyanax mexicanus	Actinopterygii	Lighter skin	[36]	

GENE	MUTATION	SPECIES	CLASS	DERIVED PHENOTYPE	REF
K locus	CBD103^{AG23} (1 bp deletion)	<i>Canis latrans</i>	Mammalia	Darker coat	[37]
	CBD103^{AG23} (1 bp deletion)	<i>Canis lupus</i>	Mammalia	Darker coat	[37]
KITLG	<i>cis</i>-regulatory changes^{A,B}	<i>Gasterosteus aculeatus</i>	Actinopterygii	Darker gills and skin	[39]
SLC24a5	Premature stop codon^B	<i>Danio rerio</i>	Actinopterygii	Lighter skin	[41]
Pax7	<i>cis</i> -regulatory change	Lake Malawi cichlids	Actinopterygii	Dark skin blotches	[42]

^AMutations also found in laboratory mice (*Mus* sp.) [43];

^BMutations also found in humans (*Homo sapiens*), for list of gene polymorphisms that associate with human eye, skin and hair color [44].

^CIndicates mutations found to be in linkage disequilibrium.

Mutations in bold indicate homologous mutations found in different species

Species in bold indicate studies where more than one mutation was found to associate with color and linkage disequilibrium was not measured.

Appendix References

1. Gross, J.B., *et al.* (2009) A Novel Role for *Mclr* in the parallel evolution of depigmentation in independent populations of the cavefish *Astyanax mexicanus*. *PLoS Genetics* 5, e1000326
2. Mundy, N.I., *et al.* (2004) Conserved genetic basis of a quantitative plumage trait involved in mate choice. *Science* 303, 1870-1873
3. Theron, E., *et al.* (2001) The molecular basis of an avian plumage polymorphism in the wild: A *melanocortin-1-receptor* point mutation is perfectly associated with the melanic plumage morph of the bananaquit, *Coereba flaveola*. *Current Biology* 11, 550-557
4. Nadeau, N.J., *et al.* (2006) Association of a Glu92Lys substitution in *MC1R* with *extended brown* in Japanese quail (*Coturnix japonica*). *Animal Genetics* 37, 287-289
5. Takeuchi, S., *et al.* (1996) A possible involvement of melanocortin 1-receptor in regulating feather color pigmentation in the chicken. *BBA-Gene Structure and Expression* 1308, 164-168
6. Ling, M.K., *et al.* (2003) Association of feather colour with constitutively active melanocortin 1 receptors in chicken. *European Journal of Biochemistry* 270, 1441-1449
7. Doucet, S.M., *et al.* (2004) Concordant evolution of plumage colour, feather microstructure and a melanocortin receptor gene between mainland and island populations of a fairy-wren. *Proceedings of the Royal Society B: Biological Sciences* 271, 1663-1670
8. Uy, J.A.C., *et al.* (2009) Difference in plumage color used in species recognition between incipient species is linked to a single amino acid substitution in the melanocortin-1 receptor. *American Naturalist* 174, 244-254
9. Baião, P.C., *et al.* (2007) The genetic basis of the plumage polymorphism in red-footed boobies (*Sula sula*): a *Metanocortin-I Receptor (MC1R)* analysis. *Journal of Heredity* 98, 287-292
10. Våge, D.I., *et al.* (2005) Two cysteine substitutions in the *MC1R* generate the blue variant of the arctic fox (*Alopex lagopus*) and prevent expression of the white winter coat. *Peptides* 26, 1814-1817
11. Klungland, H., *et al.* (1995) The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat color determination. *Mammalian Genome* 6, 636-639
12. Nachman, M.W., *et al.* (2003) The genetic basis of adaptive melanism in pocket mice. *Proceedings of the National Academy of Sciences* 100, 5268-5273
13. Hoekstra, H.E., *et al.* (2004) Ecological genetics of adaptive color polymorphism in pocket mice: geographic variation in selected and neutral genes. *Evolution* 58, 1329-1341
14. Rieder, S., *et al.* (2001) Mutations in the agouti (*ASIP*), the extension (*MC1R*), and the brown (*TYRP1*) loci and their association to coat color phenotypes in horses (*Equus caballus*). *Mammalian Genome* 12, 450-455
15. Eizirik, E., *et al.* (2003) Molecular genetics and evolution of melanism in the cat family. *Current Biology* 13, 448-453
16. Lalueza-Fox, C., *et al.* (2007) A melanocortin 1 receptor allele suggests varying pigmentation among Neanderthals. *Science* 318, 1453-1455
17. Römpler, H., *et al.* (2006) Nuclear gene indicates coat-color polymorphism in mammoths. *Science* 313, 62

18. Våge, D.I., *et al.* (1999) Molecular and pharmacological characterization of dominant black coat color in sheep. *Mammalian Genome* 10, 39-43
19. Hoekstra, H.E., *et al.* (2006) A single amino acid mutation contributes to adaptive beach mouse color pattern. *Science* 313, 101-104
20. Kijas, J.M.H., *et al.* (1998) Melanocortin receptor 1 (*MC1R*) mutations and coat color in pigs. *Genetics* 150, 1177-1185
21. Ritland, K., *et al.* (2001) Inheritance and population structure of the white-phased "Kermode" black bear. *Current Biology* 11, 1468-1472
22. Våge, D.I., *et al.* (1997) A non-epistatic interaction of *agouti* and *extension* in the fox, *Vulpes vulpes*. *Nature Genetics* 15, 311-315
23. Rosenblum, E.B., *et al.* (2004) Adaptive reptile color variation and the evolution of the *Mc1r* gene. *Evolution* 58, 1794-1808
24. Rosenblum, E.B., *et al.* (2010) Molecular and functional basis of phenotypic convergence in white lizards at White Sands. *Proceedings of the National Academy of Sciences* 107, 2113-2117
25. Nadeau, N., *et al.* (2008) Characterization of Japanese quail *yellow* as a genomic deletion upstream of the avian homolog of the mammalian *ASIP* (*agouti*) gene. *Genetics* 178, 777-786
26. Kerns, J.A., *et al.* (2004) Characterization of the dog *Agouti* gene and a *nonagouti* mutation in German Shepherd Dogs. *Mammalian Genome* 15, 798-808
27. Norris, B., and Whan, V. (2008) A gene duplication affecting expression of the ovine *ASIP* gene is responsible for white and black sheep. *Genome Research* 18, 1282-1293
28. Kingsley, E.P., *et al.* (2009) Melanism in *Peromyscus* is caused by independent mutation in *Agouti*. *PLoS One* 4, e6435
29. Linnen, C.R., *et al.* (2009) On the origin and spread of an adaptive allele in deer mice. *Science* 325, 1095-1098
30. Kuramoto, T., *et al.* (2001) Cloning of the rat *agouti* gene and identification of the rat *nonagouti* mutation. *Mammalian Genome* 12, 469-471
31. Nadeau, N.J., *et al.* (2007) Association of a single-nucleotide substitution in *TYRPI* with *roux* in Japanese quail (*Coturnix japonica*). *Animal Genetics* 38, 609-613
32. Berryere, T.G., *et al.* (2003) *TYRPI* is associated with dun coat colour in Dexter cattle or how now brown cow? *Animal Genetics* 34, 169-175
33. Schmutz, S.M., *et al.* (2002) *TYRPI* and *MC1R* genotypes and their effects on coat color in dogs. *Mammalian Genome* 13, 380-387
34. Schmidt-Küntzel, A., *et al.* (2005) *Tyrosinase* and *Tyrosinase Related Protein 1* alleles specify domestic cat coat color phenotypes of the *albino* and *brown* loci. *Journal of Heredity* 96, 289-301
35. Gratten, J., *et al.* (2007) Compelling evidence that a single nucleotide substitution in *TYRPI* is responsible for coat-colour polymorphism in a free-living population of Soay sheep. *Proceedings of the Royal Society B: Biological Sciences* 274, 619-626
36. Protas, M.E., *et al.* (2006) Genetic analysis of cavefish reveals molecular convergence in the evolution of albinism. *Nature Genetics* 38, 107-111
37. Anderson, T.M., *et al.* (2009) Molecular and evolutionary history of melanism in North American gray wolves. *Science* 323, 1339-1343
38. Haase, B., *et al.* (2007) Allelic heterogeneity at the equine *KIT* locus in dominant white (*W*) horses. *PLoS Genetics* 3, e195

39. Miller, C.T., *et al.* (2007) *cis*-regulatory changes in *Kit Ligand* expression and parallel evolution of pigmentation in sticklebacks and humans. *Cell* 131, 1179-1189
40. Moller, M.J., *et al.* (1996) Pigs with the dominant white coat color phenotype carry a duplication of the *KIT* gene encoding the mast/stem cell growth factor receptor. *Mammalian Genome* 7, 822-830
41. Lamason, R.L., *et al.* (2005) SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. *Science* 310, 1782-1786
42. Roberts, R.B., *et al.* (2009) Sexual conflict resolved by invasion of a novel sex determiner in Lake Malawi cichlid fishes. *Science* 326, 998-1001
43. Hoekstra, H.E. (2006) Genetics, development and evolution of adaptive pigmentation in vertebrates. *Heredity* 97, 222-234
44. Sturm, R.A. (2009) Molecular genetics of human pigmentation diversity. *Human Molecular Genetics* 18, R9-R17