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UNDERSTANDING EYE COLOR GENETICS IN THE DOMESTIC PIGEON

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INTRODUCTION

Across the animal kingdom, coloration and pigment pattern diversity is widespread. Despite this abundant variation, the genetic changes that lead to diversity in pigmentation are still largely unknown. Identifying specific genetic differences between different species is difficult due to the immense amount of genetic changes between them, most of which are unrelated to pigmentation. In the Shapiro lab, we use the domestic pigeon (*Columbia livia*) as a model system of genetic diversity. Through selective breeding, domestic pigeons show extensive variation in many traits, including pigmentation and pigment patterning. This variation within a single species recapitulates some of the pigment diversity seen across wild species.

One tissue that shows extensive variation in pigmentation across species is the iris of the eye (Negro et al., 2017). Iris colors can vary from bright orange, red, or blue to dark brown. The genetics of eye color variation is poorly understood, however, because while iris color varies between species, variation within a single species is less common and typically only seen in domestic animals (Negro et al., 2017).

Eye color arises from pigments in the front of the iris, which is a ring-shaped membrane that controls the amount of light that enters the eye. Iris color variation can have functional significance. For example, among mantellid frogs, tree-dwelling species are more likely to have brightly colored irises than terrestrial species, which typically have dark irises, and this bright iris coloration may serve as a defensive strategy that repels predators (Amat, 2013). Furthermore, iris color may have selective significance as there is an association between being nocturnal and having darker eye colors in owls, which is hypothesized to be beneficial for avoiding both predator and prey detection (Passarotto et al., 2018).

Pigeons are one domestic species where iris color is variable, and this makes them an ideal model to understand the genetics of iris pigmentation. There are three main iris colors that have been identified by pigeon breeders: orange, pearl (white), and bull (dark brown) (Figure 1).

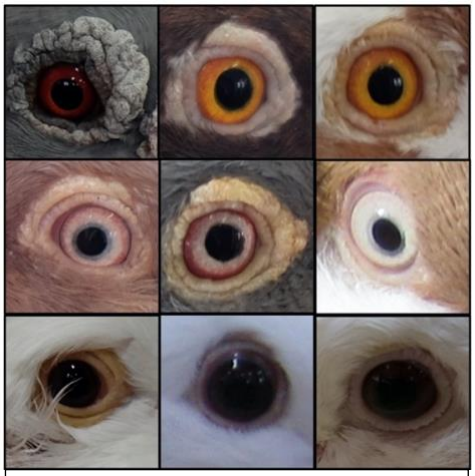

Figure 1: Eye Color variation of pigeons. Orange (top), pearl (middle), and bull (bottom).

Orange iris color is the ancestral state (Bond, 1920), and orange eyes range in shades from yellow to red, depending on the density of blood vessels in the eye (Sell, 2012). The pearl iris color is white, with tinges of pink and red from blood vessels. Lastly, the bull iris color is named based on the similarity in color to dark bovine eyes, and ranges from dark brown to almost completely black. (Huntley, 1999). Breeding experiments have shown that orange and pearl eye color are controlled by a single autosomal locus, and that the orange allele is dominant, and pearl is recessive (Bond, 1920). Less information is known about the inheritance of bull eye color. While orange and pearl irises can be found in a variety of pigeon breeds, the bull iris color is primarily found in birds with white plumage (Hollander, 1939).

Previous studies have identified two types of non-melanin pigments in the pigeon iris, guanidines and pteridines. Guanidines are whitish opaque pigments, and pteridines are yellow-orange pigment (Oliphant, 1987). In pigeons, these pigments are located on the iris stroma and stop light from getting through the eye. In orange-eyed birds, both guanidine (white) and pteridine (orange) pigments are present in the iris stroma. In white-eyed birds, only guanidine (white) pigment is present. In bull eyes, both white and orange pigments are absent in the iris stroma, so the dark melanin pigment on the inner surface of the iris can be seen. The mechanisms underlying loss of pteridine iris pigment in pearl-eyed birds or both pteridine and guanidine pigment in bull eyed birds are currently unknown. Loss could arise from defects in pigment production or failure to transport the pigment into the iris stroma, for example. To better understand the mechanisms that control iris color in domestic pigeons, we used a combination of genomic mapping and classical genetic crosses to search for loci that are associated with eye color.

RESULTS

Orange vs. Pearl Eye

To identify the genetic basis of orange and pearl eye color, we used a measure of allele frequency differentiation (pFst; Kronenberg et al., 2014) to compare the genome sequence of orange-eyed (n=16) and pearl-eyed (n=30) birds (Figure 2).

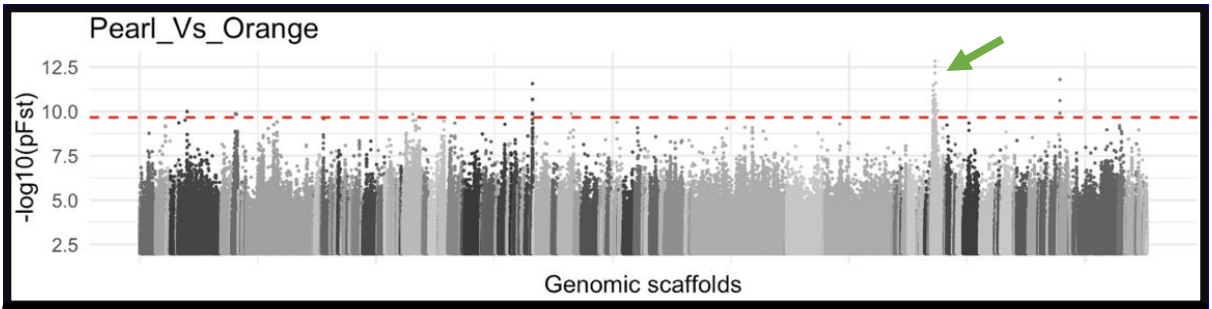

Figure 2: pFST for orange vs. pearl eye color. X axis shows genomic position, with shades of gray indicate different genomic scaffolds. Y axis shows -log10(pFST), a measure of allele frequency differentiation. Each dot represents a SNP. SNPs above the red line show significant allele frequency differentiation between orange and pearl groups.

The region with most significant differentiation (green arrow) is on genomic scaffold ScoHet5_1307, and contains multiple single nucleotide polymorphisms (SNPs) that are highly differentiated between orange and pearl groups. As pearl is recessive to orange, we expect pearl eyed birds to be homozygous for a “pearl” allele at any SNP that might cause pearl eye, and birds that are homozygous or heterozygous for the alternate allele should have orange irises. Based on this segregation pattern, were able to identify 20 SNPs that segregate perfectly between orange and pearl populations in our whole-genome sequencing data. We selected one SNP in the

middle of this region, at position ScoHet5_1307:1901234, to evaluate in a second population of 25 F2 birds from a cross between Racing Homer and Parlor Roller breeds. We found that this SNP was perfectly associated with eye color in the F2 population as well (Table 1).

	Genotype		
	C/C	C/T	T/T
Pearl eyes	11	0	0
Orange eyes	0	12	2

Table 1: Genotypes at SNP ScoHet5_1307 1901234 in Homer x Roller F2 birds.

The region of scaffold ScoHet5_1307 containing these SNPs spans multiple genes, including a promising candidate gene, Solute Carrier Family 2 Member 11-like (*Slc2a11-like)*. *Slc2a11-like* is a solute carrier that is not well studied; however, a similar solute carrier, *Slc2a11*, has been found to promote yellow pigmentation in fish scales (Kimura et al., 2014).

Bull Eye

To determine the genetic basis of bull eye color, we used quantitative trait locus (QTL) mapping in an F2 intercross derived from an orange-eyed Pomeranian Pouter and a bull-eyed Scandaroon (see Domyan et al., 2016). In this cross, F2 offspring have either orange eyes or bull eyes. We identified a single locus on linkage group 15 that is associated with bull eye color (Figure 3).

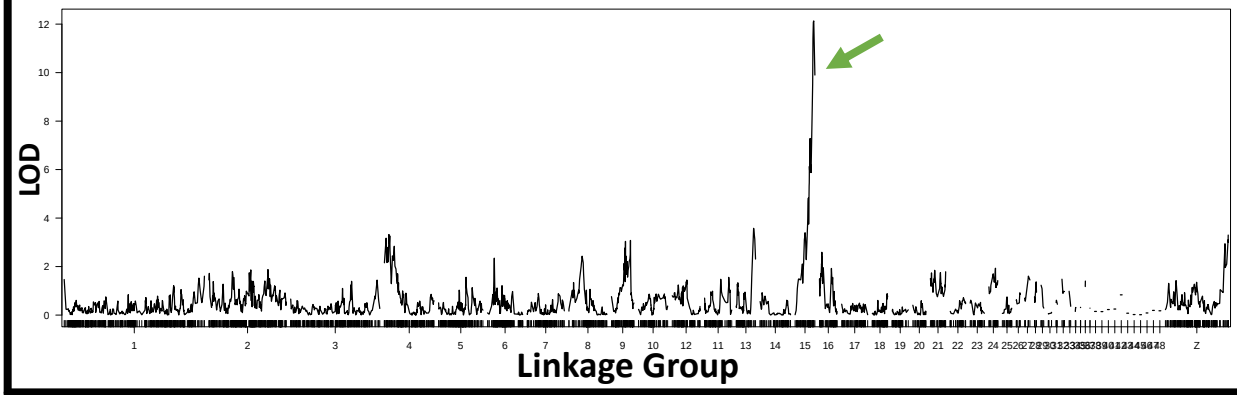

Figure 3: QTL for bull eyes. X axis indicates genomic position, ordered by linkage group. Y axis shows LOD score, a measure of association between genotype and phenotype.

Our locus (green arrow) contains an interesting candidate gene, Endothelin receptor type B2 (*Ednrb2*), which is part of a signaling pathway known to play a role in feather, skin, hair, and eye pigmentation across species (Kinoshita et al., 2014). Because breeders have noted an association between bull eye color and white plumage color in pigeons (Hollander, 1939), we wanted to evaluate the association between bull eye color and plumage pigmentation. The F2 population we used for QTL mapping has variation in a feather pigment phenotype known as piebalding, which leads to patches of non-pigmented (white) plumage. We quantified the proportion of white versus pigmented feathers in F2 birds, and tested for a relationship between eye color and plumage color in two body regions: the head and the dorsal wing. We found that bull eye is significantly associated with a higher proportion of white feathers on the head and

wings of F2 birds (Figure 4). Therefore, bull eye color is linked to feather pigmentation, suggesting that iris color and plumage color are influenced by the same genetic mechanisms.

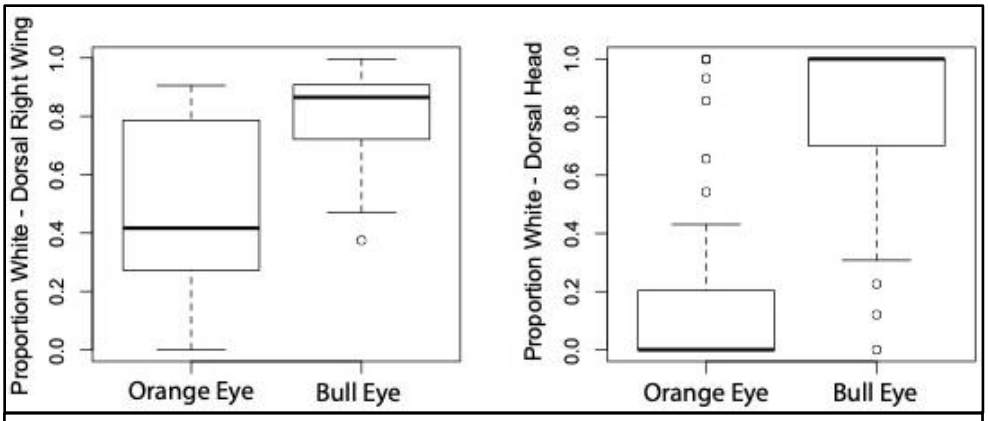

Figure 4: Association between white plumage and eye color. 2-tailed t tests compute the p-values for dorsal wing to be $p = 1.6 \times 10^{-8}$ and the head to be $p = 2.2 \times 10^{-16}$.

DISCUSSION AND FUTURE DIRECTIONS

We identified a single genomic locus that is significantly differentiated between orange eyed and pearl eyed birds, and a number of SNPs within this locus fit the known recessive inheritance pattern of pearl eye. This genomic region contains a promising candidate gene (*Slc2a11-like*), which is similar to a solute carrier that promotes yellow pigmentation in fish. As this gene is a solute carrier, pigeon eye color could change if coding or expression changes cause a failure to move orange pigment into the iris. In the future, we will determine if this gene is expressed in the pigeon eye, and if there are differences in gene expression between orange-eyed and pearl eyed birds using *in situ* hybridization and quantitative PCR.

We also identified a single locus associated with bull eyes in an F2 cross. A candidate gene in the QTL region, *Ednrb2*, has been previously shown to affect pigmentation across species. We also showed that, in this F2 cross, bull eye color is significantly associated piebalding, suggesting that a shared genetic pathway may impact both eye and feather pigment. Endothelin signaling is known to affect the development and distribution of melanin pigment in many species, but how it affects the non-melanin pigments that give rise to orange and pearl eye colors is unknown. This gene may impact eye color through altering neural crest cell migration, which is important for both eye development and plumage pigmentation, or may possibly affect the production or localization of the non-melanin pigments directly. To evaluate the role of *Ednrb2* in eye color, we first will use *in situ* hybridization in early embryos to see if *Ednrb2* is differentially expressed in the developing eye. Moreover, we are using QTL mapping in other F2 crosses to see if the breeds with bull eye color either all share the same genetic changes, or if there are many different loci that can lead to loss of iris pigmentation in bull-eyed birds.

METHODS

Eye Color Phenotyping: Eye color was determined from photographs. The sample sizes for both pFst and QTL analysis were determined by the number of photos that could be clearly scored. Birds with photos that did not clearly show eye color were not included in the data sets.

pFst: Probabilistic whole-genome scans of allele-frequency differentiation (pFst) is one of the GPAT++ tools to test for population divergence at a single nucleotide polymorphisms (SNPs) (Kronenberg et al., 2014) and is a likelihood ratio test for allele frequency differences between populations. We performed pFst analysis on genotype calls from whole-genome resequencing data of diverse domestic pigeon breeds, as previously described. (Domyan et al., 2016; Vickrey et al., 2018).

Homer/Roller Cross Genotyping: We designed PCR primers spanning intron six of our candidate gene on ScoHet5_1307, Slc2a11-like, as this region contained three SNPs associated with eye color, including ScoHet5_1307:1901234. Using these primers, we performed PCR on DNA isolated from blood samples of F2 birds in the Homer/Roller cross to amplify this target region. We purified this PCR product by gel extraction and submitted the amplified DNA for Sanger Sequencing. We then used 4Peaks (Griekspoor and Groothuis, 2015) to examine the sequencing chromatograms for each bird to determine the genotype at position ScoHet5_1307:1901234.

Genotype by sequencing: Genetic variation in the F2 Pomeranian Pouter x Scandaroon cross was characterized through Genotype by Sequencing (GBS). The GBS process uses a restriction enzyme to digest genomic DNA, then sequences size-selected fragments, as described by Domyan et al. (2016). A subset of these fragments will overlap informative single nucleotide polymorphisms (SNPs) that differ between the founders of the cross. We mapped sequencing reads to the published *C. livia* reference genome (Holt, 2018) with Bowtie2 software (Langmead, 2009). Next, we used Stacks software to identify polymorphic sites in the population and determine genotypes for each individual bird (Catchen, 2013). This told us the genotypes of each bird in the F2 cross.

QTL Mapping: The genetic basis of eye color variation was found through using Quantitative Trait Locus (QTL) mapping. QTL mapping looks for correlations between GBS data and a quantitative phenotype, or a binary trait like eye color. As the cross founders have divergent phenotypes and genotypes, the F1 offspring will be heterozygous for founder markers. After breeding the F1 generation to produce the F2 set of pigeons, these offspring will be a mix of heterozygous and homozygous genotypes because recombination has occurred. We used R/QTL software to evaluate the correlation between eye color phenotypes and marker genotypes in F2 birds (Arends et al., 2010).

Feather Pigment Phenotyping: The proportion of white feathers on the dorsal head and dorsal right wing of F2 individuals from the Pomeranian Pouter x Scandaroon cross was quantified from photographs using ImageJ.

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