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PRE-INDUSTRIAL MELANISM: THE ORIGIN, MAINTENANCE, AND GENETIC BASIS OF AN URBAN MELANIC MORPH OF THE VERMILION FLYCATCHER.

BY

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ABSTRACT

The phenomenon of urban organisms evolving dark pigmentation in response to pollution is known as industrial melanism. Industrial melanism was first noted in the Peppered Moth (*Biston betularia*) of England and after nearly 200 years of study, it is still heralded as one of the best examples of natural selection in response to anthropogenic environmental change. To date, industrial melanism has been infrequently studied in organisms other than insects. In this study, I investigated whether industrial melanism may occur in an urban population of Vermilion Flycatchers (*Pyrocephalus rubinus*). Vermilion Flycatchers are a logical choice for studying industrial melanism as the species is strikingly bright red throughout its broad range, but a sooty brown morph is abundant in the heavily polluted city of Lima, Peru. I found that the color of flycatchers is determined by a mutation in the Melanocortin-1 Receptor (MC1R) gene, which is involved in the production of melanin, or brown pigments. Specifically, I found that red flycatchers have two copies of the 'normal' MC1R gene (homozygous recessive), completely brown flycatchers have two copies of the 'mutated' MC1R gene

(homozygous dominant), and brown flycatchers with some red feathers have one copy of the 'normal' MC1R gene and one copy of the 'mutated' MC1R gene (heterozygous). Through field surveys in Lima I discovered that there were fewer heterozygotes, or brown flycatchers with some red feathers, than expected. Separately, I found that flycatchers tended to mate with individuals of similar color. Furthermore, the frequency of brown flycatchers dropped from ~60% to ~5% across the urban-suburban interface, implicating natural selection on plumage color. Using spatial statistics I found that coastal cloudiness, rather than air pollution, predicted the distribution of brown flycatchers. I conclude that natural selection relating to cloudiness and preference for mating with same-color individuals act together to maintain the brown morph in Lima. Finally, it is merely a coincidence that the brown morph occurs alongside industrial pollution in Lima; therefore, Vermilion Flycatchers don't exhibit industrial melanism, *per se*.

INTRODUCTION

I first saw a brown Vermilion Flycatcher in June 2006. I was sitting in Helen and Arturo Koenig's backyard in Miraflores with the eminent ornithologist, Dr. John P. O'Neill, when he pointed it out to me. John proceeded to explain to me his idea that the brown morph abounds in Lima because sharp-shooting, slingshot-toting boys systematically hunt the more colorful red Vermilion Flycatchers. I think he thought it was more humorous than scientific, and I enjoyed it. Although I continued to visit Lima and see the brown morph over the next few years, I didn't begin to think deeply about how unique and interesting the brown morph was until June 2011. While preparing for a three month long expedition to Peru my then undergraduate, and future Master's advisor, Chris Witt, encouraged me to pursue a project studying the evolution of the brown morph. I admit that I was hesitant to work in one of the largest cities in the world versus some exotic jungle on the eastern flank of the Andes, but I ended up seeing the exciting potential to study the Vermilion Flycatcher system.

My approach to studying the evolution of the brown morph of Vermilion Flycatchers was a combination of museum-based scientific collecting, molecular genetics, field studies, and spatial modeling. I began research on the brown morph by trying to identify its genetic basis. I took a candidate gene approach to this question and

looked for variation in the MC1R gene that was associated with the brown morph. Looking for variation in MC1R was a natural first choice given the abundant literature implicating MC1R as the cause of melanism in various vertebrates. I discovered that a nonsysonymous single nucleotide polymorphism (SNP) perfectly predicts color morph in Vermilion Flycatchers. I used this phenotype-genotype association to survey and visually genotype flycatchers in the wild in Lima. The results of the surveys indicated selection on plumage color and a tendency of individuals to mate assortatively with respect to plumage color. Finally, I used spatial modeling to show that coastal cloudiness, not pollution, best predicts the spatial distribution of the brown morph.

I learned new skills and had to overcome some difficulties while researching the evolution of the brown morph. From a molecular genetic perspective I learned lab skills required in Sanger sequencing, as well as some basic sequence analysis such as creating haplotype networks and multiple alignments. I also learned to do basic programming in the R statistical package and use geographic information system (GIS) software. Perhaps the most challenging difficulty I faced was collecting museum specimens and associated tissue samples, and surveying flycatchers in Lima, the 5th largest metropolis in the Americas. Collecting museum specimens in urban settings required patience and persistence, but ultimately taught me about the importance of discretion. Surveying flycatchers across Lima in an efficient and safe way was a logistical challenge, yet left me with an invaluable knowledge of Lima's streets, neighborhoods, and public transportation system.

I'm pleased with the how my research has incorporated museums, molecular genetics, fieldwork, GIS, human history, and natural history to enlighten our understanding of the brown morph; however, I also realize that more work will be required to unravel the complete story of plumage color evolution in the Vermilion Flycatcher. Of particular interest are the following questions. What is the age of the SNP that causes the brown morph? What is the mechanism of selection on color morph? What is the strength of selection on color morph? Are mate preferences genetically determined and linked to the SNP that causes the brown morph, or the result of imprinting? Do brown individuals outside of Lima represent vagrants or isolated populations of the

brown morph? Some of these questions can be answered with existing samples and genomic approaches, while others would require additional sampling and field studies.

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Chapter 1

Pre-industrial melanism: The origin, maintenance, and genetic basis of an urban melanic morph of the vermilion flycatcher.

Schmitt, C. J., Montaño, E. L., McNew, S. M., Vargas C., W., Valqui, T., and Witt, C. C. In Review. *Evolution*

ABSTRACT

Industrial melanism is a celebrated evolutionary phenomenon because it demonstrates the efficacy of natural selection in response to anthropogenic environmental change. We investigated a potential instance of industrial melanism in a vertebrate, the vermilion flycatcher (*Pyrocephalus rubinus*). The species is strikingly bright red throughout its broad range, but a sooty brown morph is abundant in the heavily polluted city of Lima, Peru. We found that plumage morph is perfectly predicted by a single nucleotide polymorphism in the MC1R gene. We quantified genotype and allele frequencies in seven neighborhoods of Lima. Within neighborhoods, there were fewer heterozygotes than expected, and surveys of mated pairs were consistent with assortative mating. The melanic allele frequency dropped from ~60% to ~5% across the urban-suburban interface, implicating strong divergent selection on plumage color. Spatial autoregressive models showed that coastal cloudiness predicted melanic allele frequency, while air pollution did not. We conclude that divergent selection and assortative mating act together to maintain the melanic allele at one extreme of a cloudiness gradient, even with no physical barriers to gene flow. The co-occurrence of industrial pollution and the melanic morph of the vermilion flycatcher is not industrial melanism, but a coincidence of human history and natural history.

INTRODUCTION

Color variation within animal populations is conspicuous to the human eye and to natural selection. After noting that a pigeon's plumage color affects its chance of survival, Darwin (1859, p. 85) wrote "I can see no reason to doubt that natural selection might be most effective in giving the proper colour to each kind of grouse, and in keeping that colour, when once acquired, true and constant." Indeed, natural selection on color

polymorphism (CP) has been implicated in local adaptation via crypsis in several cases (Nachman et al. 2003; Hoekstra et al. 2006; Steiner et al. 2007; Mullen and Hoekstra 2008; Linnen et al. 2009; Rosenblum et al. 2010; Cook et al. 2012). CPs can also influence mate choice (Cooke et al. 1995; Philips and Furness 1998), facilitate prezygotic reproductive isolation (Uy et al. 2009), and have pleiotropic effects on immunocompetence (Roulin et al. 2000, 2001; Gangoso et al. 2011; Jacquin et al. 2013; Lei, et al. 2013), reproductive strategy (Dreiss and Roulin 2010), and stress response (Almasi et al. 2010). In theory, CPs may lead to speciation (Gray and McKinnon 2007; Price 1998; Turelli et al. 2001) and bird lineages with CPs appear to have undergone accelerated diversification (Hugall and Stuart-Fox 2012).

The genetic causes of CP have been discovered for numerous animal populations (Theron et al. 2001; Nachman et al. 2003; Mundy et al. 2004; Baião et al. 2007; Kingsley et al. 2009; McRobie et al. 2009; Rosenblum et al. 2010; Gangoso et al. 2011; Johnson et al. 2012; Cibois et al. 2012). CPs are known to be associated with variants of more than 150 known genes, the best-known of which is the melanocortin 1-receptor or MC1R gene (Hubbard et al. 2010). MC1R encodes a transmembrane protein, which governs the relative production of eumelanin (black to brown) and phaeomelanin (yellow to red) pigments in melanocytes (McGraw 2006). In birds and mammals the deposition of eumelanin and phaeomelanin pigments in melanocytes is the primary determinant of overall coloration, and melanism is associated with simple Mendelian inheritance of non-synonymous single nucleotide polymorphisms (SNPs) at MC1R in several domestic (Figure 1; Takeuchi et al. 1996; Takeuchi et al. 1998, Robbins et al. 1993; Kijas et al. 1998; Rieder et al. 2001; Kerje et al. 2003; Fontanesi et al. 2006; Nadeau et al. 2006; Peterschmitt et al. 2009; Vidal et al. 2010a; Vidal et al. 2010b; Wenhua et al. 2012) and wild species (Figure 1; Valverde et al. 1995; Schiöth et al. 1999; Theron et al. 2001; Eizirik et al. 2003; Mundy et al. 2004; Nachman et al. 2003; Baião et al. 2007; McRobie et al. 2009; Uy et al. 2009; Gangoso et al. 2011; Cibois et al. 2012; Johnson et al. 2012; Guernsey et al. 2013).

In at least a few cases, melanic CPs resulting from single nucleotide variation at MC1R appear to have been favored by natural selection (Roulin et al 2000, 2001; Nachman et al. 2003; Steiner et al 2007; Mullen and Hoekstra 2008; Janssen and Mundy

2013; Linnen et al 2013). Theory suggests that assortative mating, apostatic selection, positive frequency-dependent selection, heterosis, divergent selection, or any combination of these mechanisms could maintain CP (Gray and McKinnon 2007). Among birds, a melanic CP in chestnut-bellied monarchs appears to be maintained by assortative mating and it may permit adaptive avoidance of hybridization, but it is unknown whether it has any ecological function (Uy et al. 2009). In parasitic jaegers, divergent selection appears to have driven the local proliferation of melanism in nesting regions that have high population density (Janssen and Mundy 2013).

Vermilion flycatchers *Pyrocephalus rubinus* are characterized by the male's bright red plumage and the female's gray and pinkish-red plumage across their wide distribution from the southwestern United States to Argentina (Fitzpatrick 2004). A striking melanic CP discovered by Charles Darwin (Gould 1838) occurs in vermilion flycatcher populations of coastal Peru. The distribution of the melanic CP is restricted to coastal lowlands of western Peru, but melanics are rare outside of the capital city of Lima (Schulenburg et al. 2010). Melanic vermilion flycatchers reach their peak frequency in Lima's urban center where they occur alongside smaller numbers of non-melanic flycatchers.

Lima is among the most populous metropolitan areas in the Americas with a population of over 10 million and notoriously polluted air. One popular hypothesis holds that urban pollution in Lima exerts selection in favor of 'industrial melanism' (*sensu* Cook et al. 2012) in vermilion flycatchers (van Grouw and Nolazco 2012). However, Darwin discovered the dark morph of the vermilion flycatcher in 1835, more than a century before the onset of intense air pollution in Lima. This suggests that Lima's pre-industrial environment may have been equally amenable to the melanic CP. The historical record may explain why Lima's natural environment is distinct from other areas along the Peruvian coast. In 1535, an indigenous leader tricked Francisco Pizarro into founding Lima in a uniquely humid and cloudy location, after which local people "danced with delight while exclaiming that the odious Spaniards would perish from mold" (translated from Maisch 1936). As indigenous peoples clearly knew, an inversion layer at Humboldt Current-Andes interface results in coastal mist and cloud cover that is locally concentrated in Lima (Capel Molina 1999). Accordingly, the gradient in

cloudiness across the periphery of Lima provides another potential adaptive explanation for the restriction of melanism to the urban center.

In this study, we tested whether melanism in vermilion flycatchers of Lima is associated with variation at the MC1R gene. Our discovery of a single diagnostic MC1R allele enabled us to distinguish melanic individuals that were homozygous or heterozygous on the basis of their plumage. This allowed us to visually survey the spatial distribution of melanic alleles across Lima and to test a series of hypotheses concerning the origin and maintenance of melanism. First, we tested whether the geographic cline in allele frequency was consistent with neutral diffusion or spatially varying selection. Second, we tested whether coastal cloudiness or air pollution could explain the modern distribution of the melanic allele. Finally, we tested for non-random local distributions of MC1R genotypes and mated pairs in order to evaluate mechanisms of natural selection that are operating currently.

METHODS

Is MC1R associated with melanism?

We conducted all analyses in the R programming language (R Core Team 2012) unless otherwise noted. We sequenced both alleles of the MC1R coding region for 39 red and 17 brown individuals from the frozen tissue collection of the Museum of Southwestern Biology, University of New Mexico (Appendix A). We extracted total genomic DNA from pectoral muscle tissue using QIAGEN DNeasy extraction kits (QIAGEN, Valencia, California) and amplified MC1R using primers MSHR9 and MSHR72 from Mundy et al. (2004). PCR products were visualized on a 1% agarose gel and cleaned with Exo-Sap-It (USB, Cleveland, OH). We sequenced diploid PCR products using BigDye 3.1 chemistry and read sequences with an ABI 3130 automated sequencer (Applied Biosystems). We aligned sequences by eye using Sequencher 4.7 (Genecodes, Ann Arbor, Michigan) and scored unambiguous double peaks on chromatograms as heterozygous sites. We tested for an association between MC1R genotype and color morph using Fisher's exact test on a 3x3 contingency table.

What is the spatial distribution of MC1R alleles in Lima?

A perfect association between MC1R genotype and plumage coloration allowed us to visually genotype birds in the wild. We surveyed, visually genotyped, and

determined sex of 691 flycatchers in the city of Lima and surrounding environs May-August 2011. We surveyed urban parks, neighborhoods, slums, agricultural areas and riparian habitats within a ~60 km radius of central Lima. Dangerous neighborhoods in inner-city Lima (*tugurios*) and peripheries (*barriadas* or *pueblos jovenes*) posed a logistical constraint that led to some sampling gaps (Chambers 2005, Plöger 2012). We calculated genotype and allele frequencies in seven neighborhoods, or subpopulations, delimited by geographic continuity. A small number of melanic individuals could not be visually genotyped (7.6%) and we assigned them as heterozygous/homozygous dominant according to the observed ratio of these genotypes in each subpopulation. We visualized melanic allele frequency across Lima using an inverse distance-weighted heat map created in ArcGIS 10 (ESRI, Redlands, California).

Are MC1R clines neutral or shaped by migration selection-balance?

Prior to European colonization the distribution of vermilion flycatchers in Lima was fragmented and restricted to narrow riparian corridors in the Chillon, Rimac, and Lurin river valleys. Given that the contemporary distribution of melanism is centered on the lower Rimac valley we assumed that the melanic allele originated and rose to high frequency (>50%) in this historically isolated valley, then spread north to the Chillon valley and south to the Lurin valley following human expansion. Prior to European colonization the distribution of vermilion flycatchers in Lima was fragmented and restricted to narrow riparian corridors in the Chillon, Rimac, and Lurin river valleys. Given that the contemporary distribution of melanism is centered on the lower Rimac valley the melanic allele originated and rose to high frequency (>50%) in this pre-historically isolated valley, then spread north to the Chillon valley and south to the Lurin valley following expansion of irrigation by indigenous humans. Accordingly, we simplified sampling across urban-rural gradients in northern and southern Lima into one-dimensional transects and grouped individuals into 5 km bins. We estimated cline shape to determine whether clines are consistent with neutral diffusion after secondary contact or migration selection balance (Barton and Hewitt 1985).

Barton and Hewitt's (1985) neutral diffusion model predicts that the cline in melanic allele frequency would decay over time since secondary contact. Alternatively, migration-selection balance would maintain a steep cline in melanic allele frequency over

time. We used the collection date of the oldest known museum specimen of a vermilion flycatcher that was heterozygous for the melanic allele (1835; see Results, below) as the minimum time-since-contact between red and melanic populations. We then tested whether contemporary cline widths are sufficiently broad to have resulted from neutral diffusion following secondary contact, or whether they are sufficiently narrow to implicate migration-selection balance.

Following previous authors (Barton and Hewitt 1985; Szymura and Barton 1986; Cheviron and Brumfield 2009), we defined cline center as the location along each transect where melanic allele frequency shifts most rapidly, and cline width as the inverse of the cline slope at the cline center. We defined the asymptotic frequencies on the left and right sides of the cline center according to the prevailing melanic allele frequency in central and suburban Lima, respectively. We estimated cline center (c), cline width (w), and the asymptotic frequency on the left and right side of the center (P_L and P_R) using maximum-likelihood in ClineFit 2.0a under the following parameters: 4000 parameter tries per annealing step, 4000 replicates, 200 replicates between saves (Porter et al. 2007). For each cline parameter estimated we also estimated two log-likelihood support limits (ln $Lmax$ – 2), which are analogous to 95% confidence intervals (Edwards 1972).

We estimated the time since a proposed contact between the red and melanic morph under the same null model of neutral diffusion (Barton and Hewitt 1985). We also created 95% confidence intervals of time-since-contact under the null model using the 95% confidence intervals of cline width estimated in ClineFit 2.0a, conservative 1-km root-mean-squared dispersal distances, and a generation-time of one year. If Darwin's 1835 heterozygous specimen were older than the 95% confidence intervals under the null model, we could reject the null hypothesis of neutral diffusion at $\alpha \leq 0.05$.

What aspects of Lima's light environment explain the distribution of melanic alleles?

We compiled data on five environmental variables associated with light-environment across Lima. First, we obtained 25 maps of small particulate pollution isobars from reports of the National Meterological and Hydrological Service of Peru, representing the years 2004-2013. We digitized these maps by hand (ArcGIS files to be deposited on Dryad) and we averaged them to produce a 10-year average pollution index. We created an inverse distance-weighted heat map of pollution index in ArcGIS to

visualize pollution across the distribution of vermilion flycatchers in Lima.

Next, we obtained cloud property data from the NASA MODerate Resolution Imaging Spectroradiometer (MODIS) instrument. The wide observation swath (2330km) and high temporal frequency (at least 2 passes a day at the equator) for 36 spectral bands of the MODIS instrument on the Terra and Aqua satellites have provided near daily global coverage observations since 2000. The MODIS atmosphere science team develops and distributes a suite of validated, science quality products including a cloud product (MOD06_L2), containing both cloud fraction and cloud optical thickness data sets at 1km and 5km spatial resolutions respectively (Menzel et al. 2010). The MODIS algorithm takes advantage of a CO_2 slicing technique measuring the partial absorption of radiation in several infrared bands, each being sensitive to different levels of the atmosphere. A cloud fraction is determined by the relationship between the fractional transmittance of radiance from a cloud versus a clear sky. The effective cloud amount (thickness) is based on the estimates of cloud top pressure (i.e. height) and increasing radiative absorption as the atmosphere becomes more opaque (i.e. cloudy) (Platnick et al. 2003).

Daily data from the Aqua satellite were obtained for the study area to produce a five-year series of average monthly cloud optical thickness and cloud fraction maps (2007-2012; 57 of 60 months). We produced an annual average data set as well as three-month (June-August) average data set centered on the period of peak cloudiness in the austral winter to assess the strong seasonal variation seen in the data (data to be deposited on Dryad). We observed strong multicollinearity among all cloud variables (Appendix B) and reduced them into linearly uncorrelated principal components. The first principal component, PC1, explained 98.7% of cloud variation and had negative loadings for each cloud variable (Appendix C). Hence we used -(PC1) as a cloudiness index, hereafter referred to as "cloudiness."

We tested for relationships between melanic allele frequency and average pollution index or cloudiness using spatial autoregressive (SAR) models. SAR models are particularly important for analyzing ecological and geographic data because they use spatial weight matrices to correct for the fact that observations from adjacent localities are often spatially autocorrelated, or more similar than expected by chance alone. Spatial

autocorrelation violates the assumption of independently distributed errors in regression analysis, causing inflated type one errors and model significance. Spatial autocorrelation can also shift model coefficients, biasing the importance of explanatory variables and complicate hypothesis testing (Legendre 1993; Kissling and Carl 2008; Dormann et al. 2007).

We corrected for spatial autocorrelation at varying spatial scales using spatial weights matrices based on 550, 200, and 10 nearest neighbors in the program GeoDa (Anselin et al. 2006). Weights based on 550, 200, and 10 nearest neighbors correct for broad scale, intermediate, and fine scale spatial autocorrelation, respectively. We used the spdep package (Bivand 2013) to find the maximum-likelihood estimates of SAR error models, which are the most reliable form of SAR models (Kissling and Carl 2008). We selected the best models from our SAR models in two steps. First, we quantified the amount of spatial autocorrelation in model residuals using Moran's I (Moran 1950) calculated with the package ncf (Bjørnstad 2005). Moran's I values range from -1 to 1 where -1 indicates perfect spatial dispersion, 1 indicates perfect spatial separation, and 0 indicates a random spatial distribution. Accordingly, models with Moran's I values close to 0 appropriately correct for spatial autocorrelation. We compared Moran's I values for each model and only considered models that minimized autocorrelation (i.e. Moran's I values close to 0). Next, we took an information theoretic approach with correction for small sample size (AIC$_c$) to select the best model that passed the previous step (Burnham and Anderson 2002). Model selection using AIC$_c$ is analogous to using AIC and we ranked all models within 4 AIC$_c$ units of the top model. Additionally, we discarded models for which nested subsets of the parameters had better AIC$_c$ scores (Arnold 2010).

Are MC1R genotypes in Hardy-Weinberg Equilibrium?

We compared observed numbers of MC1R genotypes to expectations under Hardy-Weinberg Equilibrium (HWE). We used Pearson's Chi-Square test to detect deviations from HWE and considered two potential sources of bias. First, we considered if accidental misidentification of a small proportion of heterozygous melanics as homozygous could bias HWE tests. We systematically misidentified heterozygotes in our dataset to identify if HWE tests were robust to varying levels of misidentification. Second, we considered if combining geographically structured subpopulations baised

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HWE tests via the Wahlund effect (Wahlund 1928). To test for a Wahlund effect we grouped individuals into varying numbers of subpopulations (3-26) and considered the relationship between heterozygote deficiency (expected number of heterozygotes minus observed number of heterozygotes, divided by the expected number of heterozygotes) and the area of each subpopulation.

Do color morphs and genotypes mate assortatively?

We observed individuals in mated pairs and recorded the numbers of each genotype-genotype combination. A male and female were considered a mated pair if they were observed foraging and associating in close proximity, displaying, nest building, copulating, or attending young at a nest. We tested for assortative mating among phenotypes and genotypes using Fisher's exact test on 2x2 and 3x3 contingency tables respectively in each subpopulation as well as all subpopulations pooled together. Finally, we combined our pooled results with those of van Grouw and Nolazco (2012) and tested for assortative mating among phenotypes using Fisher's exact test on 2x2 contingency tables.

RESULTS

Genetics of plumage differences

We sequenced ~800 base pairs of the MC1R coding region and identified eight synonymous and four nonsynonymous substitutions among all individuals sampled. One nonsynonymous SNP was comprised of a change from G to A at nucleotide position 274 causing a glutamate to lysine substitution at amino acid position 92 (hereafter E92K; Figure 1; Appendix D). The E92K substitution was perfectly associated with plumage morph (Figure 2; Fisher's exact test, $p=2.2 \times 10^{-16}$) and defines a MC1R haplotype that is unique to melanic flycatchers (Figure 3).

The melanic allele (E92K) is not completely dominant over the red allele and homozygous dominant individuals are solid sooty brown while heterozygous individuals are sooty brown with one or more red feathers in males, and red tinged flanks or vents in females (Appendix E, panels B-E). Homozygous recessive individuals of each sex are typical of the species outside of Lima (Appendix E, panel A). Hence we conclude that the single specimen that Darwin collected in 1835 was a heterozygote because it was described by Gould (1838) as "All the plumage chocolate-brown, tinged with red, the

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latter colour predominating on the forehead and lower part of the abdomen."

Cline analysis

Melanic genotypes and alleles were concentrated in the city center where they reached frequencies of ~65% and ~55% respectively (Figure 4). Typical red plumage remained far more abundant at the peripheries of the urban zone, despite that no barriers to gene flow were evident. Clines in melanic allele frequency estimated in Clinefit were narrow and cline width was estimated to be 6.88 and 15.68 km in northern and southern Lima, respectively (Table 1; Figure 5). We estimated the time since secondary contact under the null model to be 7.53 years (95% confidence interval= 0.02–22.35 years) in northern Lima and 39.13 years (95% confidence interval= 6.37×10^{-5} – 91.75 years) in southern Lima. The age of Darwin's melanic specimen was older and did not fall within the 95% confidence interval of time since secondary contact under the null model in northern or southern Lima. We reject the null hypothesis that neutral diffusion of melanic alleles following secondary contact resulted in the steep melanic allele frequency cline in northern and southern Lima

Genotype-environment correlations

SAR models that corrected for spatial dependence at fine-scales (i.e. those using spatial weights based on 10 nearest neighbors) had the least amount of spatial autocorrelation (Appendix F). Accordingly, we present the results of the SAR error models incorporating a 10-nearest-neighbor spatial weights matrix. Among 10-nearest-neighbor SAR models, parameter estimates illustrate that both cloudiness and average pollution index were positively correlated with melanic allele frequency (Table 2). The best-supported model based on AICc model selection included cloudiness as a statistically significant predictor of melanic allele frequency (p=0.03). Average pollution index was not a statistically significant predictor variable (p=0.37). The model with both pollution and cloudiness resulted in higher AICc score (ΔAICc=3.14) than cloudiness alone and was not considered (Arnold 2010). A visual examination of melanic allele frequency was consistent with the SAR model results, showing more concordance with cloud variables (peaks in northern, central, and southern Lima) than with pollution (peaks in northern and eastern Lima; Figure 6).

Tests of Hardy-Weinberg Equilibrium

A dearth of heterozygotes was present in subpopulations that cover very small areas, and the magnitude of heterozygote deficiency didn't increase substantially with larger subpopulations (Appendix G). We choose to group individuals into 7 subpopulations for the final HWE tests in order to minimize Wahlund effects without compromising sample size. The observed proportions of each MC1R genotype differed significantly from expectations under HWE in each of the seven subpopulations (Table 3). We observed that a deficit of heterozygotes was responsible for deviations from HWE in all comparisons (Table 3). Finally, we found that the observed deviation from HWE in each of the seven subpopulations was large enough to have been robust to modest levels of misidentification of heterozygous melanics (Appendix H).

Tests of assortative mating

We observed 85 mated pairs in six of seven subpopulations in Lima. Sixty-eight mated pairs (80%) had both members of the pair with matching MC1R genotypes ("same-genotype"; Table 4) and 76 (89%) had both members of the pair sharing similar melanic or non-melanic plumage phenotypes ("same-color"; Appendix I). Observed genotype combinations departed significantly from expectations under random mating in one of six subpopulations and when all subpopulations were pooled together (Table 4). Observed color combinations did not depart significantly from expectations under random mating in any subpopulations, but differed significantly in the direction of same-color pairs when all subpopulations were pooled together (Appendix I). Post hoc power analyses of same-color mated pairs indicated limited statistical power to detect departures from random mating in subpopulations and that we would need to double sample sizes in most subpopulations to have sufficient statistical power to detect departures from random mating at $\alpha=0.05$ (Appendix J). When we pooled all subpopulations together, as well as when we included data from van Grouw and Nolazco (2012), power analyses showed that statistical power was adequate to detect departures from random mating at stringent significance levels ($\alpha<0.0001$; Appendix J). Finally, we considered the possibility that spatial clusters of dark or light alleles within subpopulations could potentially have biased our results towards positive assortative mating. We don't think that this is the case because a detailed examination of same-genotype and same-color mated pairs showed that many pairs were found in the immediate neighborhoods of potential mates of the

opposite genotype and color, respectively (Appendix K).

DISCUSSION

MC1R variants perfectly predict melanism

A perfect association between MC1R genotype and phenotype indicates that MC1R contributes to the distinctive melanic morph of vermilion flycatchers in Lima. The same E92K substitution is associated with melanism in five other species (Figure 1; mice, Robbins et al. 1996; chicken, Takeuchi et al. 1996; bananaquit, Theron et al. 2001; japanese quail, Nadeau et al. 2006; and tahiti reed-warbler, Cibois et al. 2012), and in vitro experiments in mice have shown that the substitution constitutively activates the MC1R protein (Robbins et al. 1996). Among wild birds, melanism-causing alleles at MC1R are either dominant (bananaquit, Theron et al. 2001; chestnut-bellied monarch, Uy et al. 2009; Eleonora's falcon, Gangoso et al. 2011; Tahiti reed-warbler, Cibois et al. 2012) or incompletely dominant (snow goose and parasitic jaeger, Mundy et al. 2004; red-footed booby, Baião et al. 2007; gyrfalcon, Johnson et al. 2012). The melanic MC1R allele in vermilion flycatchers is incompletely dominant (heterozygotes are melanic with few red or red tinged feathers). Although the structure of melanin and the biochemical mechanism of melanin deposition in feathers has not been studied in this species, it is likely that melanic plumage is the result of an added layer of melanin, which masks the typical red carotenoids of feathers.

MC1R clines indicate migration-selection balance

Steep genetic clines can be fleeting under neutral diffusion of alleles following secondary contact (Haldane 1948), or they can be stable when maintained by migration-selection balance (Barton and Hewitt 1985). Neutral diffusion of alleles would provide a poor explanation for the clines in MC1R allele frequency in vermilion flycatchers. Under the null model of neutral diffusion, time since secondary contact would have been unrealistically recent, especially considering historical specimens that demonstrate mixing of melanic and non-melanic alleles at least as far back as Darwin's discovery of this population in 1835 (Gould 1838). We conclude that clines in melanic allele frequency are maintained by migration-selection balance; however, assortative mating could also slow the diffusion of melanic alleles. Although hypothetical demographic events may have caused the observed spatial structuring of melanic alleles by chance

processes such as allele surfing (Edmonds et al.2004; Klopfstein et al. 2006; Antoniazza et al 2014), and these could be tested in the future using population genomic data, we consider these special explanations to be less likely than spatially variable selection. Furthermore, any historical population structure must be exceedingly recent because a range-wide phylogeography study of this species found no mtDNA population structure across Lima, the entire west slope of Peru, and the Marañon Valley (O. Carmi, A. Jaramillo, C. C. Witt, and J. P. Dumbacher, unpublished manuscript), an area encompassing four recognized subspecies (Dickinson 2003).

Coastal cloudiness predicts the distribution of melanic alleles

Coastal cloudiness predates the city's founding by Francisco Pizarro in 1535 (Capel Molina 1999), and we can only assume that the pattern of cloudiness has been stable for the intervening centuries. Our spatial models based on NASA MODIS data from 2007-2012 showed that coastal cloudiness predicts the modern day distribution of the melanic allele in Lima. Strong clines in melanic allele frequency coinciding with clines in cloudiness are suggestive of local adaptation via disruptive selection. Air pollution does not seem to explain additional variation in contemporary melanic allele frequency. This makes sense in light of that fact that many historic specimens of melanic vermilion flycatchers from Lima predate the mid-20th century boom in industrial and automobile pollution (Appendix L). Although it would be useful to test for changes in the distribution of melanism since industrialization, the numerous pre-industrial flycatcher specimens from Lima were likely not collected randomly with respect to color morph, and we found that locality data on their specimen labels was insufficiently precise (Appendix L).

Assortative mating contributes to deviations from Hardy-Weinberg Equilibrium

Departures from HWE in Lima are suggestive of assortative mating. Specifically, a dearth of heterozygotes suggests that assorative mating, disruptive selection, and/or underdominance may be occurring. Among our sample of mated pairs (n=85) we found statistically significant departures from random mating. We found that 88% of mates were of similar color and 80% were of similar genotype. Van Grouw and Nolazco (2012) did not find a statistically significant pattern of assortative mating (n=42 pairs), although they did find a tendency for dark birds to pair with other dark birds. When we combined

our data with van Grouw and Nolazco's (2012) we found that same-colored pairs still predominated (80%; Appendix I). One caveat is that extra pair copulation, known to occur in a Mexican population of vermilion flycatchers (Ríos-Chelén et al. 2008), could alter the relationship between pair-combination frequency and mating frequency, particularly if it occurs systematically with respect to color. Nonetheless, the modest statistical evidence of assortative mating provided by the pair-combination frequencies corroborated our finding of fewer than expected heterozygotes in each subpopulation. Even imperfect assortative mating could help to maintain locally adaptive alleles (Kirkpatrick 2000), in this case by effectively slowing the rate of movement of non-melanic alleles into the city center.

Mechanisms of selection and implications for speciation

We suggest that melanism in vermilion flycatchers is an example of abrupt geographic variation following Gloger's rule (Zink and Remsen 1986); populations exposed to more humid climates have most likely evolved darker plumage due to natural selection. An intriguing aspect of this particular case is that sexual selection via assortative mating may be effectively slowing gene flow across the environmental gradient and increasing the frequency of melanic homozygotes, the individuals that experience the greatest degree of fitness advantage. It has been demonstrated previously that natural and sexual selection acting on the same trait can slow gene flow and facilitate ecological speciation (Turelli et al. 2001; Gray and McKinnon 2007).

There are four plausible, non-mutually exclusive mechanisms of melanic fitness advantage in this case. First, melanism has been shown to facilitate cryptic background matching on dark substrates (rock pocket mouse, Nachman et al. 2003; peppered moth, Cook et al. 2012) and melanic plumages may be similarly cryptic in Lima's muted light-environment. Second, feathers are broken down by keratinolytic bacteria (Williams et al. 1990, Burtt and Ichida 1999) and melanism has been shown to reduce degradation by bacteria (Goldstein et al. 2004, Gunderson et al. 2008). Feather-degrading bacteria from humid regions and darker plumaged birds has been shown to break down feathers more rapidly than feather-degrading bacteria from dry regions and lighter plumaged birds, under controlled conditions (Burtt and Ichida 2004). Hence it is plausible that feather-degrading bacteria comprise a more severe ecological pressure in the more humid

environment of central Lima, causing melanism to be locally advantageous for its protective effect. Third, the ability of melanins to absorb and convert the sun's photo energy to heat may be locally advantageous in central Lima because it can increase metabolic economy or other aspects of thermoregulation in birds and other animals (zebra finch, Hamilton and Heppner 1967; *Colias* butterflies, Watt 1968; zebra finch, Heppner 1970; pierid butterflies, Kingsolver 1987; two-spot ladybird beetles, De Jong et al. 1996). Fourth, melanism may have pleiotropic effects that increase immune activity (eleonora's falcon, Gangoso et al. 2011; barn owl, Roulin and Ducrest 2011) or resistance to haemosporidian parasites (rock pigeon, Jacquin et al. 2013; black sparrowhawk, Lei et al. 2013), either of which could be locally advantageous in Lima's cool, humid environment. To distinguish among these causes, experimental work is needed to test for functional differences between melanics and non-melanics in the core and periphery of Lima.

Conclusion

We showed that a single locus causes a melanic CP in vermilion flycatchers of Lima and genotypes can be assessed using plumage or a nonsynonymous MC1R SNP. Our surveys of mated pairs suggest that assortative mating contributes to the maintenance of melanism in Lima's cloudy urban center. The local concentration of this trait at frequencies exceeding 50% is remarkable because of direct proximity to nearly pure red populations on the city's northern and southern margins, despite no evident barriers to dispersal. The most likely explanation for the peculiar distribution of melanic vermillion flycatchers is natural selection favoring melanism in the dim light environment that is created by locally intense coastal cloudiness. Thus, an apparent case of industrial melanism is a coincidence of human and natural history. Air pollution cannot explain the pre-industrial presence of melanic vermilion flycatchers, and our results indicate that particulate pollution and the resulting diminution of sunlight does not explain any residual variation in the modern distribution of melanic alleles. This example shows how natural selection exerted by the physical environment and sexual selection, exhibited as a preference for sameness, can act in combination to maintain a distinct, highly local phenotype without physical barriers to geneflow.

ACKNOWLEDGEMENTS

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Chapter 1

FIGURES

Figure 1. Amino acid sequence variation associated with melanism in bird and mammal orthologs of MC1R. Variants are coded according to whether they have been implicated in eumelanin or pheomelanin coloration in avian and mammalian species. Figure based on previous review of avian (Mundy 2005) and mammalian (Majerus and Mundy 2003) MC1R diversity.
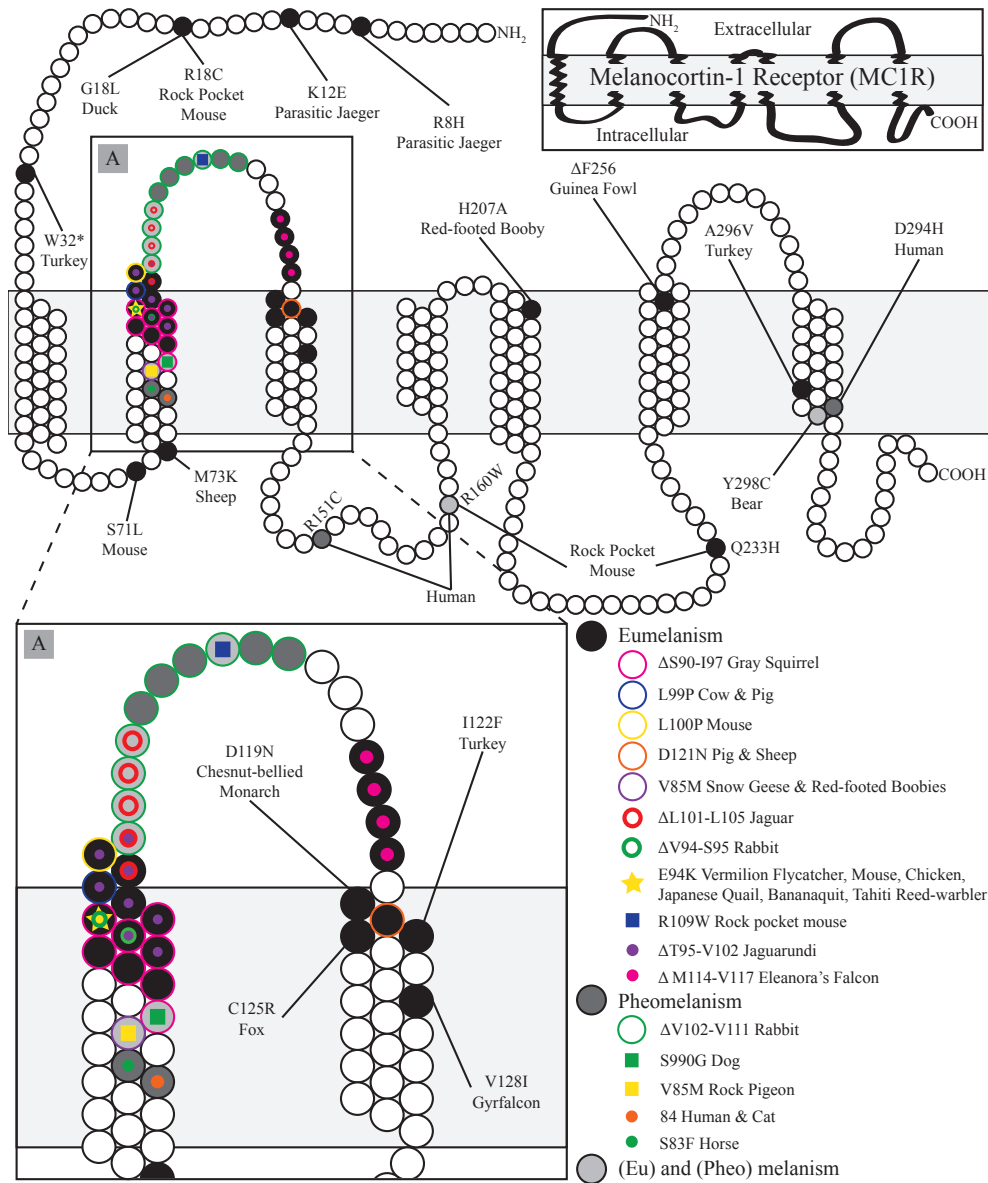
Figure 2. Genotype-phenotype associations between MC1R alleles and plumage morphs (Fisher's exact test, p= 2.2×10^{-16}). Watercolor paintings by CJS depict typical plumages, but photographs of each individual can be viewed in the online specimen database accessible through the links provided in Appendix A.

Flycatcher phenotype

MC1R genotype			
Glu/Glu	39	-	-
Glu/Lys	-	14	-
Lys/Lys	-	-	3

Figure 3. Median-joining network of vermilion flycatcher MC1R haplotypes created in Network v4.6.1.1 with standard settings (http://www.fluxusengineering.com). Haplotypes were inferred in DnaSP v5 (Librado and Rozas 2009). Circles represent different sampled haplotypes and are sized in proportion to their frequencies, and distances between haplotypes are proportional to the number of base pair differences. Haplotypes are colored according to color morph and subspecies. The E92K nonsynonymous substitution associated with plumage color is labeled.
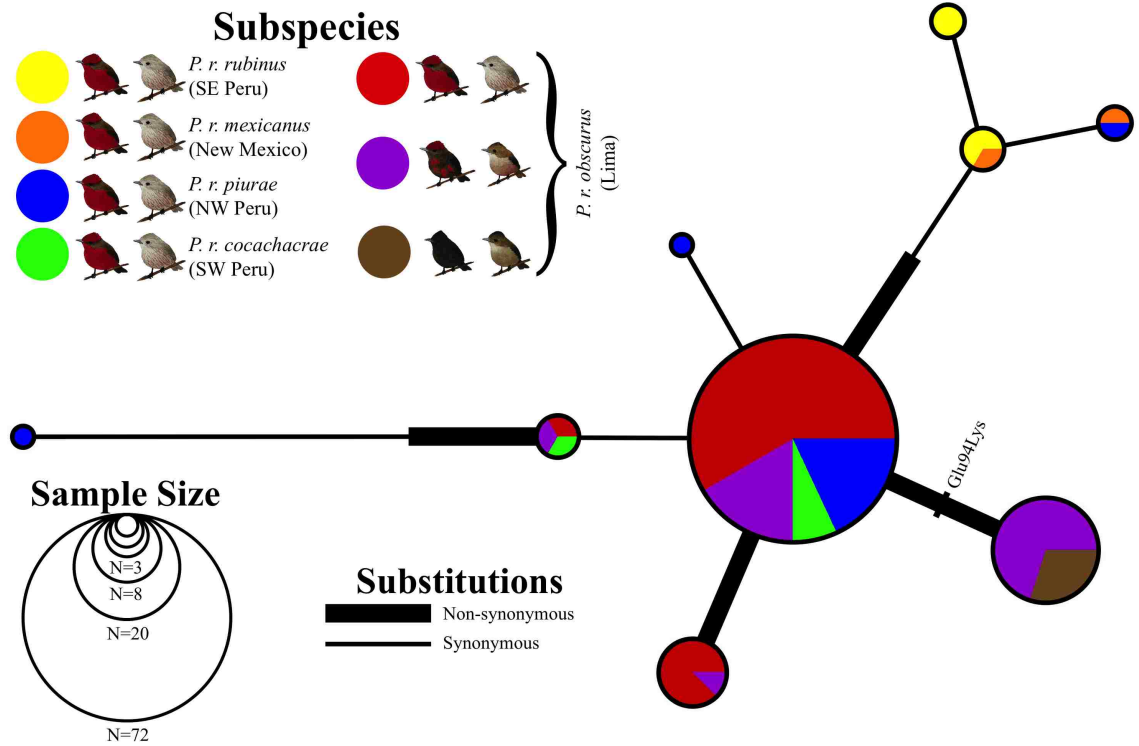
Figure 4. The estimated distribution of vermilion flycatchers in Lima (light gray shading) is shown in relation to South America (right, red shading). Shading on map indicates melanic allele frequency for each subpopulation surveyed. Pie graphs of genotype frequencies are shown for each subpopulation.
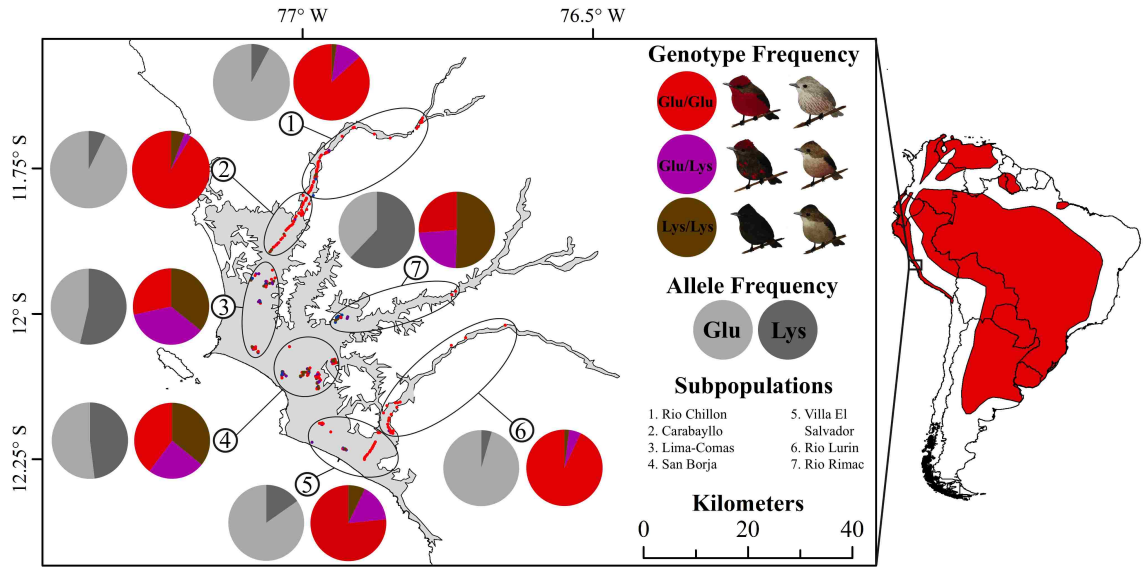
Figure 5. Clines in melanic allele frequency plotted against geographic distance along transects in northern and southern Lima (panels A and B). Solid lines are maximum-likelihood cline shape estimates for melanic allele frequency and gray shading represents log-likelihood support limits (ln *Lmax* – 2). The location of transects are indicated on a map of Lima where light gray shading indicates contemporary flycatcher habitat and dark gray shading indicates estimated pre-industrial flycatcher habitat (panel C).
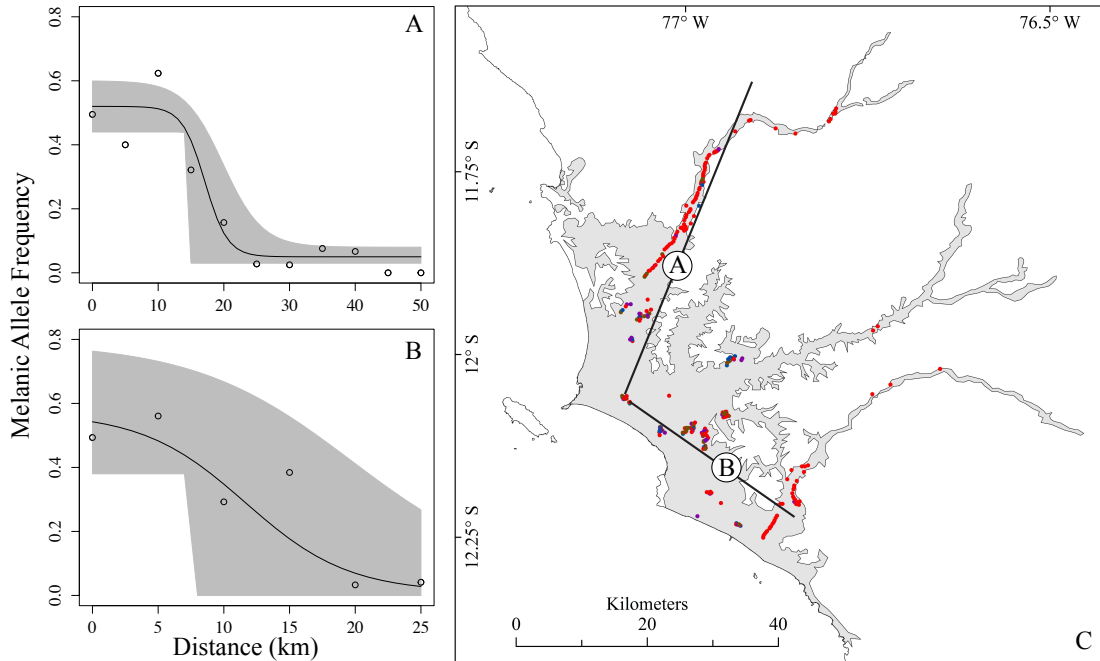
Figure 6. Maps of melanic allele frequency and each environmental variable are shown across the extent of the estimated distribution of available habitat for the vermilion flycatcher in Lima. The geographic distribution of the upper two quartiles of melanic allele frequency (panel A) is shown in relation to the overlap between the upper two quartiles of melanic allele frequency and the upper two quartiles of pollution (panel B) and cloudiness (panel C).
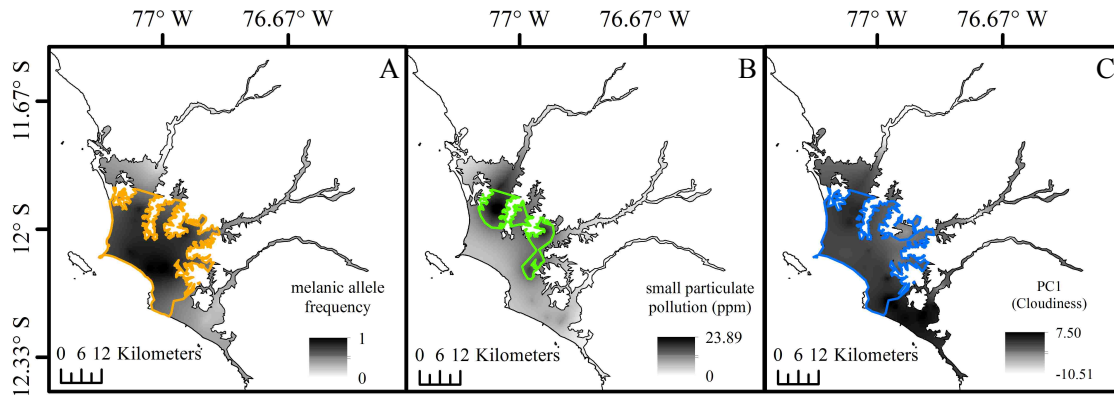
Chapter 1

TABLES

Table 1. Maximum-likelihood estimates of center (c), width (w), asymptotic frequency at the left side of cline in urban Lima (pL), and right side of cline in suburban Lima (pR) for clines in melanic allele frequency along the northern and southern transect, respectively. Two log-likelihood support limits (ln $Lmax - 2$), which are analogous to 95% confidence intervals (Edwards 1972), are reported in parentheses.

	Northern cline	Southern cline
c	17.11 (14.41–19.89)	11.64 (7.59–19.73)
w	6.88 (0.31–11.85)	15.68 (0.02–24.01)
pL	0.52 (0.44–0.60)	0.57 (0.38–0.79)
pR	0.05 (0.03–0.08)	0.01 (0–0.05)
AIC_c	432.29	555.96

Table 2. Results of 10 nearest neighbor SAR error models for predicting melanic allele frequency (P = average pollution index; C= cloudiness). The model with C and P was not considered because it resulted in a higher AICc score (ΔAICc=3.14) than the model with C alone (Arnold 2010). Model selection with AIC and AICc produced identical results.

mode type	model	df	p value	parameter estimate	AICc	ΔAICc	AICc weight
SAR	C	687	0.03	1.40×10^{-2}	467.78	0	0.86
SAR	P	687	0.37	6.08×10^{-3}	471.47	3.69	0.14

Table 3. Chi-square tests between observed and expected (under Hardy-Weinberg equilibrium) numbers of each genotype among individuals visually genotyped in subpopulations of Lima (n=691). Note that the observed numbers are not always integers because dark plumaged individuals that could not be genotyped were allocated to genotype categories based on the subpopulation-specific frequencies of known genotype birds.

	Observed			Expected			df	χ^2	p-value
	AA	AG	GG	AA	AG	GG			
Carabayllo	5.33	2.67	84	0.48	12.37	79.15	1	56.60	5.33×10^{-14}
San Borja	59.49	40.51	66	38.31	82.87	44.82	1	43.38	4.99×10^{-11}
Rio Lurin	2.29	5.71	106	0.23	9.82	103.95	1	19.94	8.00×10^{-6}
Villa el Salvador	5.54	12.46	59	1.80	19.94	55.26	1	10.83	9.98×10^{-4}
Lima-Comas	36.5	36.5	29	29.39	50.72	21.89	1	8.02	4.62×10^{-3}
Rio Rimac	21.21	9.79	11	16.26	19.76	6.02	1	10.69	1.08×10^{-3}
Rio Chillon	2.17	10.83	85	0.59	13.99	83.42	1	4.99	2.54×10^{-2}

Table 4. Counts of each genotype-genotype mate class and p-values for Fisher's exact test in 7 subpopulations. Males are listed first in each mate class.

	GG-GG	GG-AG	GG-AA	AG-GG	AG-AG	AG-AA	AA-GG	AA-AG	AA-AA	p-value
San Borja	5	1	1	1	1	0	1	0	5	0.03
Carabayllo	13	0	0	0	0	0	0	1	0	0.07
Rio Chillon	11	1	0	2	1	0	0	0	0	0.37
Lima-Comas	1	1	0	0	4	1	0	5	3	0.40
Lurin	8	0	0	0	0	0	1	0	0	1.00
Pachacamac	16	1	0	0	0	0	0	0	0	1.00
Total	54	4	1	3	6	1	2	6	8	3.26×10^{-11}

CONCLUSION

Darwin's legacy endures in many ways, to be sure; however, underappreciated amongst them is a single specimen he collected in Lima, Peru. One unknown day in July 1835 Darwin recounts that he "went out with some merchants to hunt in the immediate vicinity of the city," and we can only assume that it was on this occasion that he discovered the melanic moprh of the Vermilion Flycatcher and had the foresight to document its existence by preserving one specimen. Darwin himself didn't acknowledge the importance of his discovery saying, "our sport was very poor," and he could only have imagined how his theory of evolution by natural selection has influenced my study of melanism in Vermilion Flycatchers.

180 years later after Darwin's serendipitous discovery I uncovered the genetic basis of melanism in Vermilion Flycatchers. I found that that a single mutation in the MC1R gene causes some flycatchers to be red and others to be brown. Surveys of mated pairs suggest that flycatchers in Lima preferentially mate with same-color individuals, which partially explains why melanism is restricted to Lima's cloudy urban center. The high concentration of melanism in central Lima is remarkable because there are no barriers to dispersal between the city center and populations of nearly pure red individuals on the city's northern and southern margins. I found that natural selection associated with coastal cloudiness, but not pollution, favors melanism in the urban center of Lima. Accordingly, Vermilion Flycatchers don't exhibit industrial melanism and are instead an example local trait variation that is maintained by the combined forces of natural and sexual selection. In conclusion, I showed that a single gene causes melanism in Vermilion Flycatchers and that selection maintains melanism at one end of a cloudiness gradient despite physical barriers to geneflow.

APPENDIX A

Samples with links to Genbank and the ARCTOS database. Genotype refers to the nucleotides found at position 274 of the MC1R gene on each of the two alleles.

Country	Department	Specific locality	Genotype	Catalog number and ARCTOS link	Genbank accession
Peru	Ancash	Huarmey	GG	MSB:Bird:34216	pending
Peru	La Libertad	1.6 km SE Huacapongo	GG	MSB:Bird:34682	pending
Peru	La Libertad	1.6 km SE Huacapongo	GG	MSB:Bird:34714	pending
Peru	Lambayeque	ca. 9.8 km N. of Olmos	GG	MSB:Bird:33770	pending
Peru	Lambayeque	ca. 9.8 km N. of Olmos	GG	MSB:Bird:33794	pending
Peru	Lambayeque	ca. 9.8 km N. of Olmos	GG	MSB:Bird:33800	pending
Peru	Lambayeque	ca. 9.8 km N. of Olmos	GG	MSB:Bird:33807	pending
Peru	Lambayeque	ca. 9.8 km N. of Olmos	GG	MSB:Bird:33853	pending
Peru	Lambayeque	ca. 9.8 km N. of Olmos	GG	MSB:Bird:33867	pending
Peru	Lima	Lurigancho-Chosica	AA	MSB:Bird:31417	pending
Peru	Lima	Lurigancho-Chosica	GG	MSB:Bird:31432	pending
Peru	Lima	Lurigancho-Chosica	AA	MSB:Bird:32963	pending
Peru	Lima	Lurigancho-	AG	MSB:Bird:32	pending

		Chosica		977	
Peru	Lima	Lurigancho-Chosica	AG	MSB:Bird:32987	pending
Peru	Lima	Lurigancho-Chosica	AG	MSB:Bird:32997	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36389	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36391	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36392	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36393	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36394	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36395	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36396	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36397	pending
Peru	Lima	Antioquia; Lurin valley	GG	MSB:Bird:36399	pending
Peru	Lima	Antioquia; Lurin valley	GG	MSB:Bird:36400	pending
Peru	Lima	Antioquia; Lurin valley	GG	MSB:Bird:36401	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36402	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36403	pending

Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36404	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36405	pending
Peru	Lima	Pachacamac; Lurin valley	AG	MSB:Bird:36406	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36407	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36408	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36410	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36411	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36412	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36413	pending
Peru	Lima	Pachacamac; Lurin valley	GG	MSB:Bird:36414	pending
Peru	Lima	Pachacamac; Lurin valley	AG	MSB:Bird:36415	pending
Peru	Lima	Pachacamac; Lurin valley	AG	MSB:Bird:36416	pending
Peru	Lima	Santa Rosa de Quives; Chillon valley	AG	MSB:Bird:36417	pending
Peru	Lima	Santa Rosa de Quives; Chillon valley	AG	MSB:Bird:36418	pending
Peru	Lima	Carabayllo;	AG	MSB:Bird:36	pending

		Chillon valley		419	
Peru	Lima	Carabayllo; Chillon valley	AG	MSB:Bird:36 420	pending
Peru	Lima	Los Olivos	GG	MSB:Bird:36 421	pending
Peru	Lima	Los Olivos	AG	MSB:Bird:36 422	pending
Peru	Lima	Lurigancho-Chosica	GA	MSB:Bird:36 424	pending
Peru	Lima	Lima	GA	MSB:Bird:36 425	pending
Peru	Lima	Santiago de Surco	AA	MSB:Bird:36 426	pending
Peru	Lima	Santiago de Surco	AG	MSB:Bird:36 427	pending
Peru	Madre de Dios	Iberia	GG	MSB:Bird:37 039	pending
Peru	Madre de Dios	Iberia	GG	MSB:Bird:37 052	pending
Peru	Tacna	11.0 km NE Locumba	GG	MSB:Bird:35 038	pending
Peru	Tacna	11.0 km NE Locumba	GG	MSB:Bird:35 055	pending
Peru	Tacna	Curibaya Pampa	GG	MSB:Bird:35 468	pending
United States	New Mexico	San Marcial	GG	MSB:Bird:26 864	pending

Scatterplot matrix illustrating multicolinearity among cloud variables (CT= annual cloud optical thickness; CT1= June-August cloud optical thickness; CF= annual cloud fraction; CF2= June-August cloud fraction). Values in boxes above the diagonal represent Pearson's product-moment correlation coefficients.
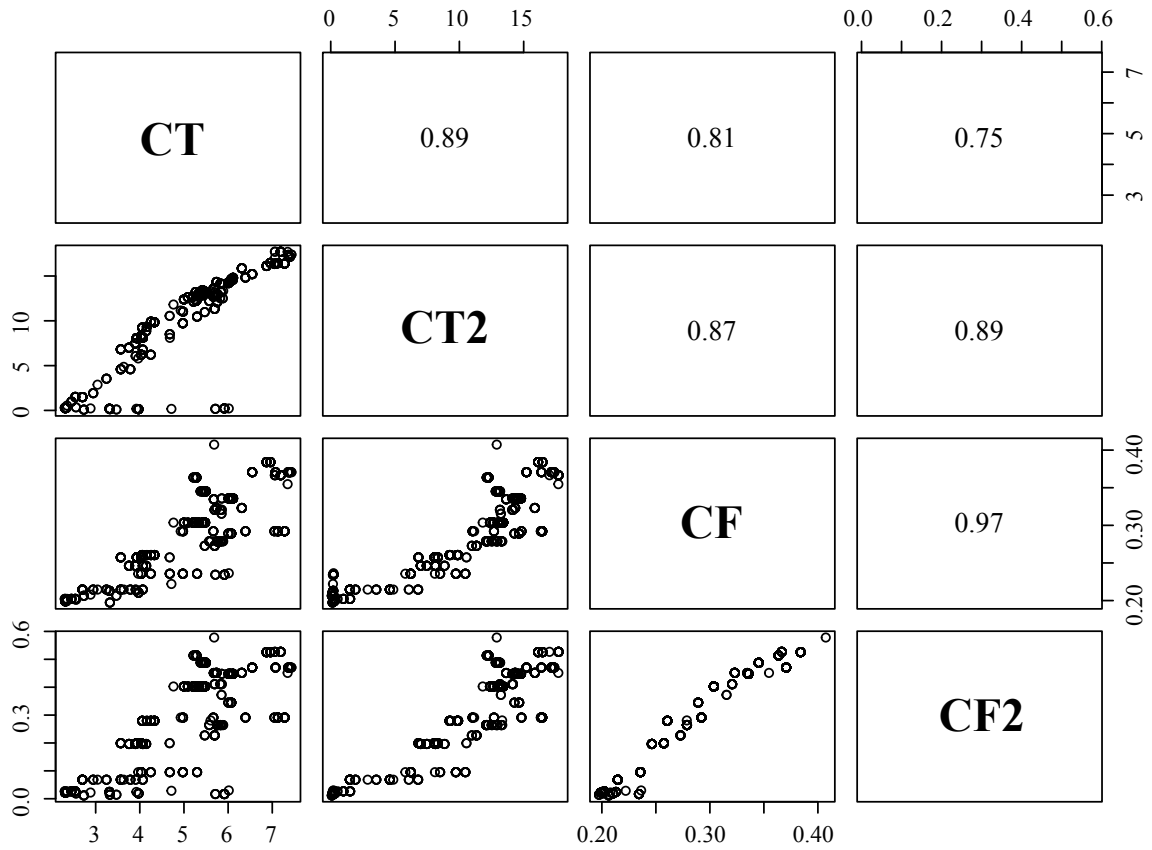
APPENDIX C

Results of principle component analysis presented as percent variance explained and principle component loadings for each cloud variable (CT= annual cloud optical thickness; CT1= June-August cloud optical thickness; CF= annual cloud fraction; CF2= June-August cloud fraction).

|	PC1	PC2	PC3	PC4
Variance explained	98.67%	1.31%	0.02%	<0.00%
CT loadings	-0.23	-0.97	-0.02	0.01
CT2 loadings	-0.97	0.23	-0.03	0.00
CF loadings	-0.01	-0.01	-0.31	-0.95
CF2 loadings	-0.03	0.03	-0.95	0.31

APPENDIX D

MC1R protein alignment for vermilion flycatcher and six other bird species with melanism-causing mutations at MC1R.

| taxa	84	85	86	87	88	89	90	91	92	93	94
Vermilion Flycatcher (wild type/red)	L	V	S	I	S	N	L	A	E	M	L
Vermilion Flycatcher (eumelanic/brown)	-	-	-	-	-	-	-	-	K	-	-
Tahiti Reed-warbler (wild type)	-	-	-	-	-	-	-	-	-	-	-
Tahiti Reed-warbler (eumelanic)	-	-	-	-	-	-	-	-	K	-	-
Bananaquit (wild type)	-	-	-	-	-	-	-	-	-	-	-
Bananaquit (eumelanic)	-	-	-	-	-	-	-	-	K	-	-
Rock Pigeon (wild type)	-	Ÿ	-	V	-	-	-	V	-	T	-
Rock Pigeon (eumelanic)	-	M	-	V	-	-	-	V	-	T	-
Snow Goose (wild type)	-	-	-	V	-	-	-	V	-	T	-
Snow Goose (eumelanic)	-	M	-	V	-	-	-	V	-	T	-
Japanese Quail (wild type)	-	-	-	V	-	-	-	-	-	T	-
Japanese Quail (eumelanic)	-	-	-	V	-	-	-	-	K	T	-
Chicken (wild type)	-	-	-	V	-	-	-	-	-	T	-
Chicken (eumelanic)	-	-	-	V	-	-	-	-	K	T	-

Photographs of five vermilion flycatchers collected for this study, showing representative plumages. (A, MSB:Bird:36389; B, MSB:Bird:36422; C, MSB:Bird:36426; D, MSB:Bird:36424; E, MSB:Bird:36427).
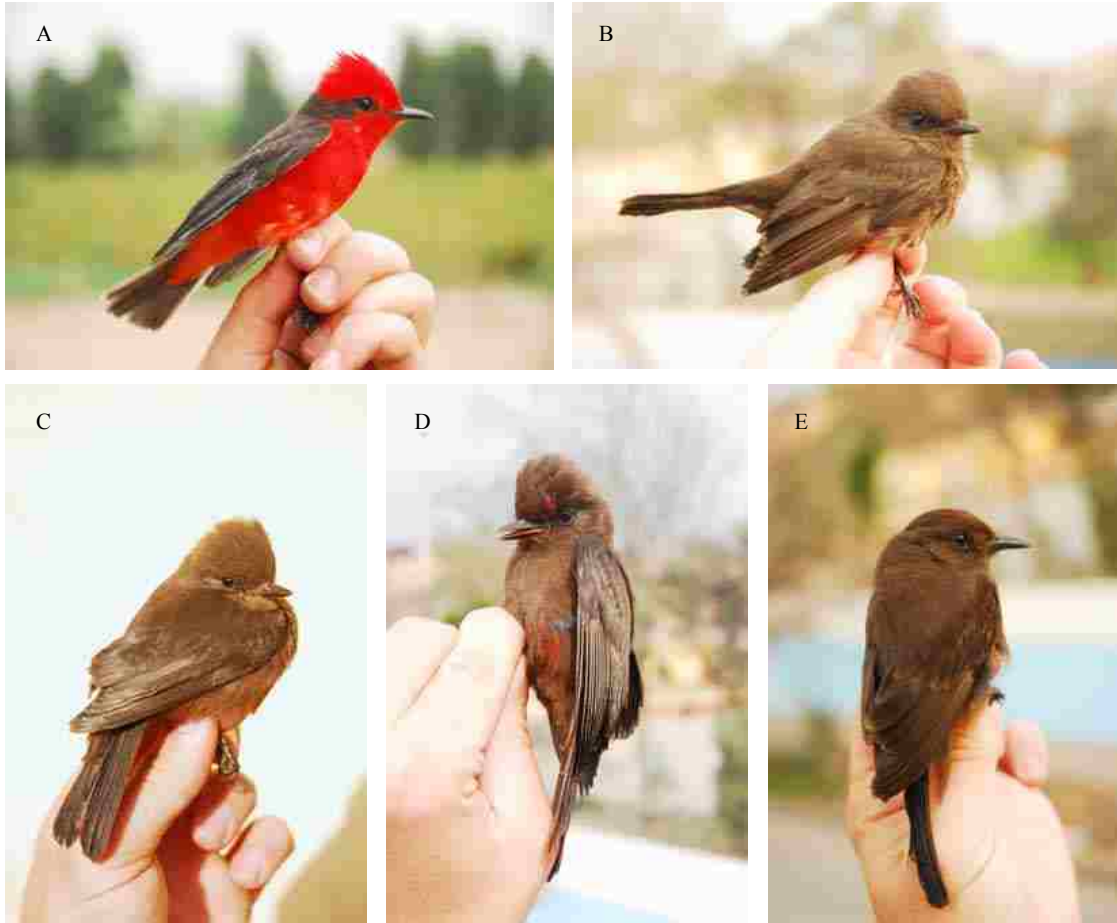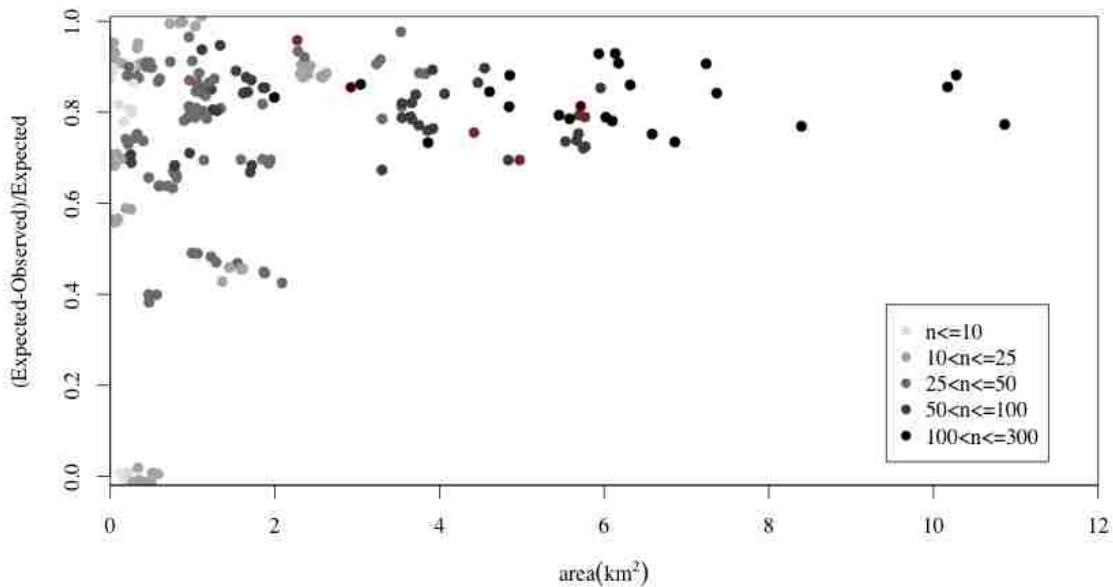
APPENDIX F

Correlograms of OLS and SAR error models with cloudiness, pollution index, and pollution+cloudiness as independent variables. Moran's I is a measure of spatial autocorrelation and a value of zero indicates no spatial autocorrelation. OLS as well as SAR models incorporating weights matrices based on many nearest neighbors (i.e. 500 and 200 nearest neighbors) had nonzero Moran's I values indicating spatial autocorrelation. Accordingly, we discarded these models from spatial modeling because spatial autocorrelation violates the assumption of independently distributed errors in regression analysis.
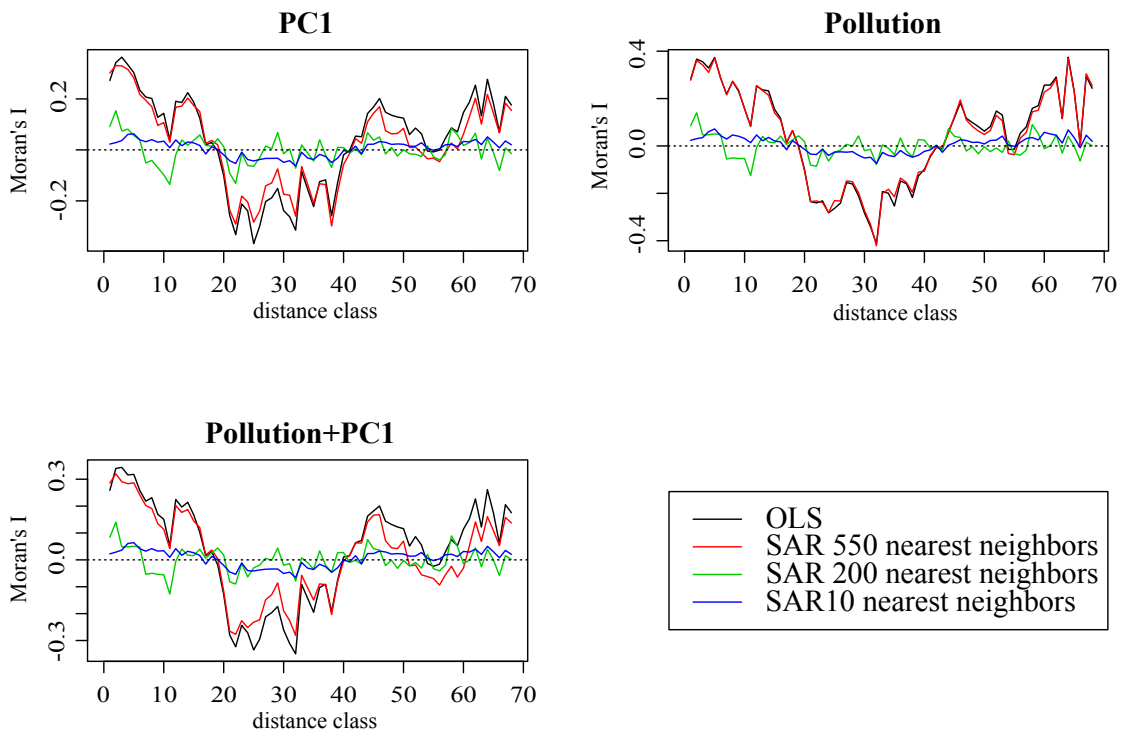
The Wahlund effect lowers heterozygosity when geographically structured subpopulations are combined and can bias tests of HWE. We plotted heterozygote deficiency (expected number of heterozygotes minus observed number of heterozygotes, divided by the expected number of heterozygotes) in each subpopulation against the area of each subpopulation. The sample size of each subpopulation is indicated by shading. A dearth of heterozygotes (values close to 1) was evident in subpopulations that cover very small areas, and the magnitude of heterozygote deficiency didn't increase substantially with larger subpopulations. Some small subpopulations (bottom left) had small expected-observed discrepancies (values close to 0); however, this was driven by small sample sizes (<25). We choose to group individuals into 7 subpopulations (indicated in red) for the final HWE tests in order to minimize Wahlund effects without compromising sample size.

The effect of misidentifying melanics as homozygotes. The observed deviation from HWE in each of the seven subpopulations was large enough to have been robust to modest levels of misidentification of heterozygous melanics.
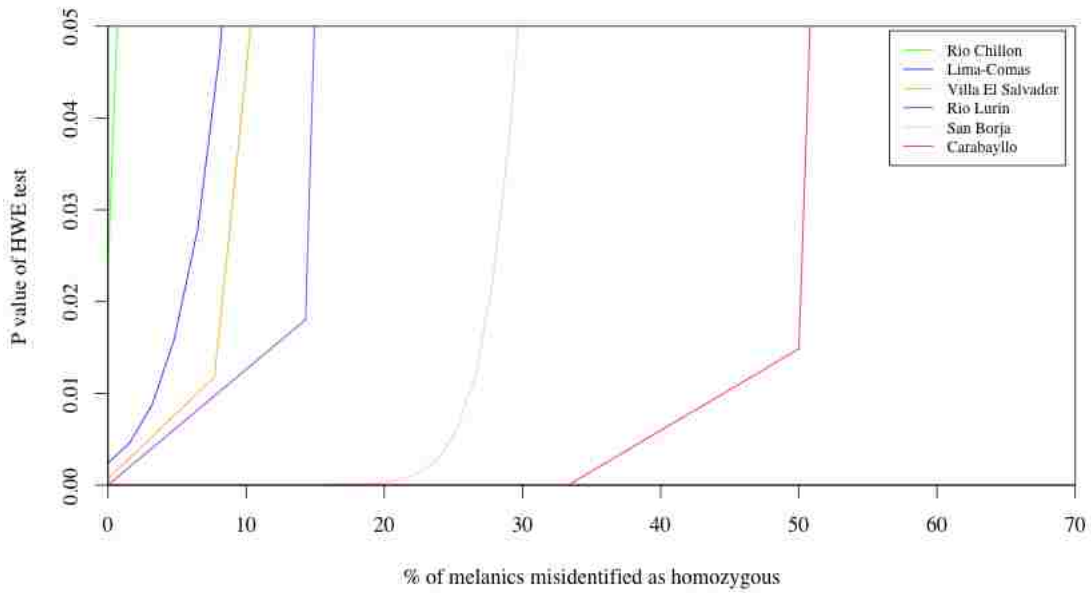
Counts of each phenotype-phenotype mate class and p-values for Fisher's exact test on 2x2 contingency table in 6 subpopulations and all subpopulations pooled together. We also present previous data from van Grouw and Nolazco (2012) as well as their data combined with ours. Males are listed first in each mate class.

|	red-red	red-brown	brown-red	brown-brown	p-value
Carabayllo	13	0	0	1	0.07
San Borja	5	2	2	6	0.10
Lima-Comas	1	1	0	13	0.13
Rio Chillon	11	1	2	1	0.37
Lurin	8	0	1	0	1.00
Pachacamac	16	1	0	0	1.00
Subpopulation total	54	5	5	21	6.68×10^{-12}
van Grouw and Nolazco	8	10	6	18	0.16
This study + van Grouw and Nolazco	62	15	11	39	4.83×10^{-11}

Post hoc power analyses of Fisher's exact test to detect assortative mating. Graphs show the statistical power of Fisher's exact test to detect assortative mating given sample sizes in the study (left side) and hypothetically larger sample sizes. In each graph statistical power is the probability of detecting assortative mating at varying p-values (colored lines).
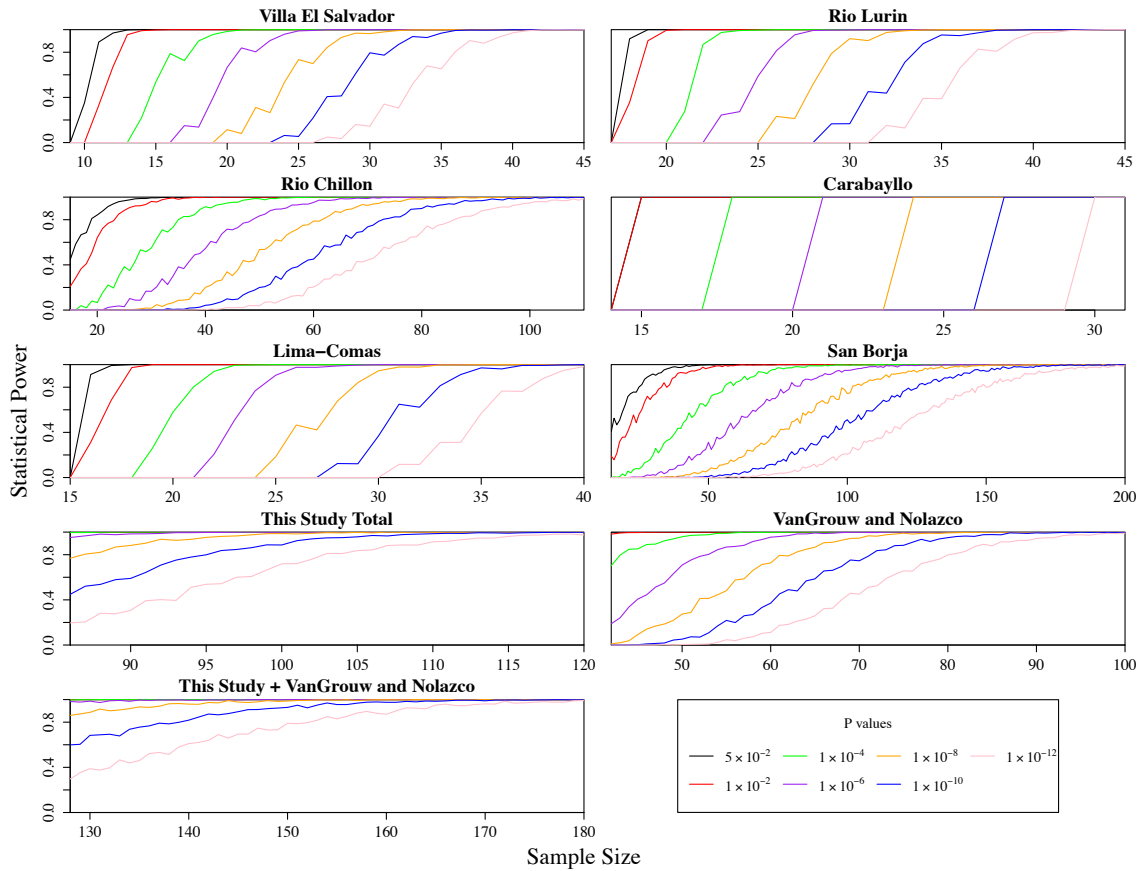
Fine-scale spatial evidence of assortative mating. Same-color mated pairs were found in neighborhoods that contained ample individuals of the opposite color. Locations of all same-color mated pairs found in neighborhoods with birds of opposite color are indicated in relation to the study area on upper-left panel (A-K). Black triangles represent mated pairs and same-color mated pairs are surrounded by circles (500 meter radius) colored according to mate combination (red=red-red mate pair; brown=brown-brown mate pair). Points represent other birds occurring in each neighborhood and are colored according to their genotype (red=CC, purple=TC, blue=brown individual with unknown genotype, brown=TT).
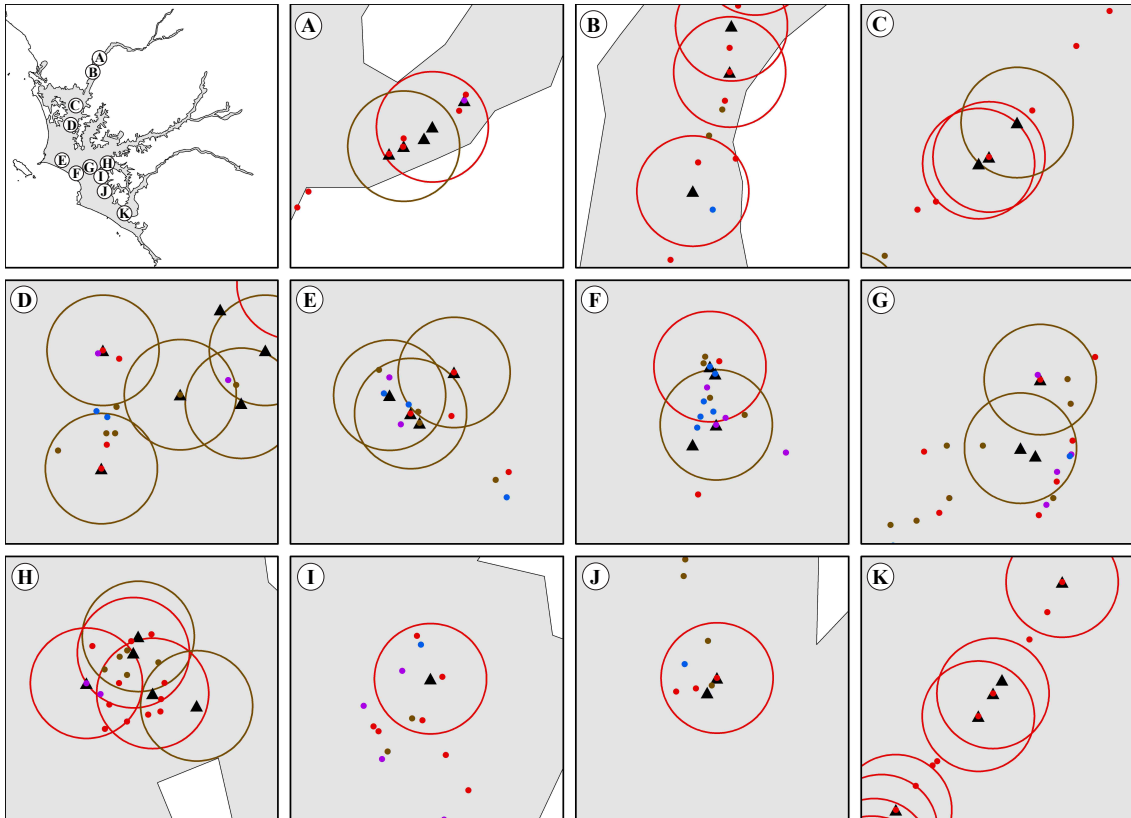
APPENDIX L

Some of the historic specimens of melanic vermilion flycatchers from Lima, Peru, that document the high pre-industrial frequency of the melanic allele. These specimens were queried from museums in the United States using the ORNIS database and melanism was confirmed with digital photographs.

Institution	Catalog number	Preparator/collector	Locality	Date
National Museum of Natural History	USNM 14366	T. R. Peale	Callao, Peru	-
National Museum of Natural History	USNM 14382	T. R. Peale	Callao, Peru	-
National Museum of Natural History	USNM 14986	T. R. Peale	Callao, Peru	-
National Museum of Natural History	USNM 39908	W. Nation	Lima, Peru	-
National Museum of Natural History	USNM 39909	-	Lima, Peru	-
Field Museum of Natural History	FMNH 303885	T. B. Wilson	Lima, Peru	-
Univ. Michigan Museum of Zoology	UMMZ 23258	Beal and Steere	Callao, Lima, Peru	May 1873
Univ. Michigan Museum of Zoology	UMMZ 23259	Beal and Steere	Callao, Lima, Peru	May 1873
Harvard Museum of Comparative	MCZ 82939	R. H. Beck	Lima, Peru	28 January 1913

Zoology				
American Museum of Natural History	AMNH 166001	R. H. Beck	Lima, Peru	28 January 1913
American Museum of Natural History	AMNH 166002	R. H. Beck	Lima, Peru	28 January 1913
American Museum of Natural History	AMNH 166003	R. H. Beck	Lima, Peru	28 January 1913
American Museum of Natural History	AMNH 165999	R. H. Beck	Lima, Peru	7 February 1913
American Museum of Natural History	AMNH 166004	R. H. Beck	Lima, Peru	10 February 1913
American Museum of Natural History	AMNH 151922	H. Watkins	Huaral, Lima, Peru	16 December 1918
American Museum of Natural History	AMNH 151948	H. Watkins	Vitarte, Lima, Peru	20 February 1919
American Museum of Natural History	AMNH 151949	H. Watkins	Vitarte, Lima, Peru	20 February 1919
American Museum of Natural History	AMNH 151965	H. Watkins	Vitarte, Lima, Peru	20 February 1919
American Museum of Natural History	AMNH 151966	H. Watkins	Vitarte, Lima, Peru	20 February 1919

American Museum of Natural History	AMNH 151967	H. Watkins	Vitarte, Lima, Peru	20 February 1919
American Museum of Natural History	AMNH 151968	H. Watkins	Vitarte, Lima, Peru	21 February 1919
American Museum of Natural History	AMNH 151969	H. Watkins	Vitarte, Lima, Peru	21 February 1919
American Museum of Natural History	AMNH 151953	H. Watkins	Vitarte, Lima, Peru	24 February 1919
American Museum of Natural History	AMNH 151956	H. Watkins	Vitarte, Lima, Peru	25 February 1919
American Museum of Natural History	AMNH 151954	H. Watkins	Vitarte, Lima, Peru	25 February 1919
American Museum of Natural History	AMNH 151957	H. Watkins	Vitarte, Lima, Peru	26 February 1919
American Museum of Natural History	AMNH 151973	H. Watkins	Vitarte, Lima, Peru	26 February 1919
American Museum of Natural History	AMNH 151974	H. Watkins	Vitarte, Lima, Peru	26 February 1919
American Museum of Natural History	AMNH 151960	H. Watkins	Vitarte, Lima, Peru	27 February 1919
American	AMNH 151961	H. Watkins	Vitarte,	27 February

Museum of Natural History			Lima, Peru	1919
American Museum of Natural History	AMNH 151962	H. Watkins	Vitarte, Lima, Peru	28 February 1919
American Museum of Natural History	AMNH 151979	H. Watkins	Vitarte, Lima, Peru	14 March 1919
Field Museum of Natural History	FMNH 59803	J. T. Zimmer	Vitarte, Lima, Peru	26 April 1922
Field Museum of Natural History	FMNH 59804	J. T. Zimmer	Vitarte, Lima, Peru	26 April 1922
Harvard Museum of Comparative Zoology	MCZ 273999	W. Weyrauch	Lima, Peru	27 October 1941
Harvard Museum of Comparative Zoology	MCZ 273997	W. Weyrauch	Lima, Peru	November 1941