Evolution and Speciation in Birds:

Insights from Cardinals and Boobies

by

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ABSTRACT

Speciation, or the process by which one population diverges into multiple populations that can no longer interbreed with each other, has brought about the incredible diversity of life. Mechanisms underlying this process can be more visible in the early stages of the speciation process. The mechanisms that restrict gene flow in highly mobile species with no absolute barriers to dispersal, especially marine species, are understudied. Similarly, human impacts are reshaping ecosystems globally, and we are only just beginning to understand the implications of these rapid changes on evolutionary processes. In this dissertation, I investigate patterns of speciation and evolution in two avian clades: a genus of widespread tropical seabirds (boobies, genus *Sula*), and two congeneric passerine species in an urban environment (cardinals, genus *Cardinalis*). First, I explore the prevalence of gene flow across land barriers within species and between sympatric species in boobies. I found widespread evidence of gene flow over all land barriers and between 3 species pairs. Next, I compared the effects of urbanization on the spatial distributions of two cardinal species, pyrrhuloxia (*Cardinalis sinuatus*) and northern cardinals (*Cardinalis cardinalis*), in Tucson, Arizona. I found that urbanization has different effects on the spatial distributions of two closely related species that share a similar environmental niche, and I identified environmental variables that might be driving this difference. Then I tested for effects of urbanization on color and size traits of these two cardinal species. In both of these species, urbanization has altered traits involved in signaling, heat tolerance, foraging, and maneuverability. Finally, I tested for evidence of selection on the urban populations of both cardinal species and found evidence of both parallel selection and introgression between the species, as well

as selection on different genes in each species. The functions of the genes that experienced positive selection suggest that light at night, energetics, and air pollution may have acted as strong selective pressures on these species in the past. Overall, my dissertation emphasizes the role of introgression in the speciation process, identifies environmental stressors faced by wildlife in urban environments, and characterizes their evolutionary responses to those stressors.

DEDICATION

For my dog, Bear Peak Canyon, who has never let me work more than 45 consecutive minutes on this dissertation without taking a break and playing with him. Thanks for teaching me that nothing is more important than eating rocks and barking at trash cans in the sunshine, not even a doctorate degree.

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PREFACE

The speciation process underlies the great diversity of life on Earth, allowing single populations of interbreeding individuals to diverge into multiple populations that can no longer produce viable offspring together (Coyne and Orr 2004). Evolutionary biologists have characterized patterns of speciation across a myriad of taxa since Darwin first described the speciation process (Darwin 1859), including notable recent contributions that have found evidence of hybridization and introgression across taxa (Payseur and Rieseberg 2016), identified genes that defy species boundaries with putative adaptive functions (Harrison and Larson 2014, Taylor and Larson 2019), and discovered genetic variants associated with traits that define population differences (Edelman et al. 2019, Toews et al. 2016). However, many open questions still remain about how species diversify in circumstances without physical barriers to gene flow. Sympatric congeneric species present a unique opportunity to study the speciation process in its early stages (Price 2008), and contemporary technologies enable more advanced studies of niche ecology, genomic divergence, gene flow, and trait evolution, which together can provide novel insights into the speciation process.

Physical barriers to gene flow, such as a mountain range, a river, or an expanse of uninhabitable landscape, isolate populations and allow for the accumulation of incompatible genetic variants between the populations, resulting in speciation (Coyne and Orr 2004). However, highly mobile species may not ever experience complete physical isolation, and in these situations, other isolating factors such as mating preferences might play an unusually large role in the early speciation process (Friesen 2015). Therefore, an essential step in the process of identifying the mechanisms involved in the speciation of

highly mobile species is characterizing the extent of genetic isolation of populations across physical barriers and between sympatric species.

In sympatric species that hybridize but show but no evidence of introgression, selection against hybrids can play a major role in maintaining the species boundary (Coyne and Orr 2004). Disruptions to the selective environment might affect the species boundary and allow genetic exchange between the species that otherwise did not occur. One such major disruption is the rapid change to our planet's ecosystems that has resulted from human activities, including the effects of climate change, land use change, and the introduction of novel species (Asamoah 2022). Urbanization radically alters every aspect of an ecosystem, with changes to resource distributions, local climates, predation pressures, noise levels, and light environments, among others (Isaksson 2018). These massive ecological perturbations also result in changes to the evolutionary process and can alter patterns of selection on and gene flow between closely related species (Grabenstein and Taylor 2018). Selection in urban areas often acts similarly across species (Otto 2018), but even closely related species can have different responses (Caizergues et al. 2022, McNew et al. 2017). Human activities will continue to reshape the world in even more drastic ways in the future, and it is essential that we develop an understanding of how human activities affect speciation and evolution of native species to manage our ecological impacts.

Birds are an excellent study system in which to study questions of speciation in the absence of physical barriers (Price 2008). They are uniquely mobile which reduces the effect of what few barriers might prevent sympatric species from interacting (Friesen 2015). Hybridization is relatively common in birds, even between quite diverged

lineages. Bird genomes are relatively small compared to many other vertebrates (Kapusta et al. 2017), which reduces the cost of whole-genome sequencing, and reference genomes are widely available from many species across class *Aves* (Feng et al. 2020). Finally, thanks to extensive community science efforts, large datasets of location-based observations of birds that span every month of the year are publically available (Fuller 2020, Sullivan et al. 2009). In my dissertation, I studied patterns of gene flow across land barriers and species boundaries in boobies, a genus of seabirds (Family *Sulidae*, genus *Sula*). I also compared spatial distributions, morphological trait changes, and genomic patterns of differentiation and introgression in urban pyrrhuloxia (*Cardinalis sinuatus*) and northern cardinals (*Cardinalis cardinalis*), two sister species of passerines.

Of the six species of booby, three are pantropically distributed: red-footed boobies (*Sula sula*), brown boobies (*Sula leucogaster*), and masked boobies (*Sula dactylatra*) (Nelson 1978). The other three species are found along the eastern Pacific Ocean: Nazca boobies (*Sula granti*), blue-footed boobies (*Sula nebouxii*), and Peruvian boobies (*Sula variegata*). All six species display striking bare-part coloration patterns, particularly in their bills, faces, irises, and feet, and some evidence suggests that these traits are used in mate choice (Montoya et al. 2018, Torres and Velando 2008). They all breed on islands and coasts, and forage on open water (Nelson 1978). Population structure has been extensively characterized across the ranges of all three pantropical species using a mix of mitochondrial, microsatellite, and multi-locus nuclear sequence data, with populations clustering in the Pacific, Indian, and Atlantic Oceans (Morris-Pocock et al. 2010, Morris-Pocock et al. 2011, Steeves et al. 2003, Steeves et al. 2005a, Steeves et al. 2005b). Four species pairs have putative instances of a hybrid offspring (masked and brown boobies,

Richard White, pers. comm.), documented instances of hybrids (blue-footed and Peruvian, Taylor et al. 2010b, 2012a; and blue-footed and brown, Taylor et al. 2013), interspecific courting (blue-footed and Nazca, Figueroa and Stucchi 2008; masked and Nazca, T. Steeves pers. obs.), or in one case evidence of introgression (blue-footed and Peruvian; Taylor et al. 2012a).

Previous work has demonstrated that various human activities disrupt long-standing species boundaries (Grabenstein and Taylor 2018), including urbanization (Chafin et al. 2019, Grabenstein et al. 2022). Pyrrhuloxia and northern cardinals are sister species (Scott 2022) that nest in similar, often overlapping territories in the Sonoran Desert (Gould 1960) and occupy regions in and around the Tucson metropolitan area. Putative hybrids have been observed on eBird, but none have been genetically confirmed, and genetic data does not show patterns of introgression between rural populations of the two species (Kaiya Provost pers. comm.). The range of northern cardinals extends further north and includes some areas of the Phoenix metropolitan area. The two species are readily distinguished by the extent of red coloration in males, with northern cardinals nearly entirely red except for their black face masks (Halkin et al. 2021), while pyrrhuloxia have red breasts, masks, crests, wings, and tail but are otherwise grey (Tweit and Thompson 2020). Female pyrrhuloxia are similar to the male but with a buffy breast and a dark grey face mask, and female northern cardinals are similar to female pyrrhuloxia but with a bright orange bill. The bill shape also differs between the species, as pyrrhuloxia have a decurved bill much like a parrot bill but northern cardinals have a conical bill.

For the first chapter of my dissertation, I sequenced the genomes of individuals across the ranges of each booby species. I analyzed patterns of gene flow across land barriers in each pantropical species, and tested for the presence of introgression between sympatric species for which hybridization or interspecific courting has been observed.

For the second chapter of my dissertation, I modeled the distributions of *Cardinalis* species in Arizona at both the city and statewide level using community science data. I tested for differences in the environmental niche of both cardinal species across the state of Arizona and across the city of Tucson, and I aimed to identify environmental variables that may explain differences in the urban occupancies of the species.

For the third chapter of my dissertation, I collected photographs and trait size measurements of both wild-caught and museum specimens of *Cardinalis cardinalis* and *C. sinuatus* in Arizona over a 137-year period. I tested for effects of urbanization on color traits, and for effects of time and color on trait sizes of both species. I also compared between the two species to identify shared traits that have been affected by urbanization.

For the fourth and final chapter of my dissertation, I sequenced the genomes of urban and rural birds from both *Cardinalis* species. I tested for genes that are highly differentiated between urban and rural populations, and for genes that have undergone positive selection in the urban environment. I also tested for patterns of introgression in several genes that were associated with urbanization in both species.

This dissertation is an exploration of the nuances of speciation and evolution in birds. I explore patterns of gene flow in a clade of tropical seabirds with higher resolution sequence data than ever before, and test for introgression between multiple species pairs.

I also apply sophisticated spatial modeling approaches to large community science datasets to compare the effects of urbanization on patterns of urban occupancy between two *Cardinalis* species. I model morphological trait changes over more than a century and across varying degrees of urbanization, and I use whole genome sequences to investigate patterns of genetic variation in response to anthropogenic change. I present novel investigations that characterize unique and intruiging processes of speciation and evolution in birds.

CHAPTER 1

INTERSPECIFIC INTROGRESSION AND WIDESPREAD INTRASPECIFIC GENE FLOW IN A CLADE OF TROPICAL AND SUBTROPICAL SEABIRDS

Abstract

The mechanisms that restrict gene flow between populations and facilitate population differentiation and speciation vary across the tree of life. In systems where physical barriers to gene flow are dynamic over time and space, such as many marine species, genetic introgression may be a major factor in the speciation process. In sympatric species of seabirds, hybridization has been frequently observed but few studies have investigated patterns of introgression. We used whole-genome sequence data to test for interspecific introgression between five pairs of tropical and subtropical seabird species and to test for gene flow within species across major land mass barriers and ocean basins. We found evidence for introgression between: blue-footed (*Sula nebouxii*) and Peruvian boobies (*S. variegata*); masked (*S. dactylatra*) and Nazca boobies (*S. granti*); and blue-footed and Nazca boobies. We found no evidence of introgression between blue-footed and brown boobies (*S. leucogaster*), or masked and brown boobies, despite observed hybridization between these species. We also found evidence for gene flow across several major land masses in three pantropical species: red-footed (*S. sula*), brown, and masked boobies. Finally, we report mixed evidence for ancient introgression between brown boobies and the ancestor of blue-footed, Peruvian, masked, and Nazca boobies. Our work indicates (1) that interspecific introgression has shaped contemporary booby diversity in the eastern Pacific, and (2) that contemporary physical barriers to gene flow

between booby colonies are not absolute. Our findings contribute novel insights to the growing body of evidence that introgression is a widespread evolutionary process.

Introduction

The extent to which inter- and intraspecific gene flow has contributed to diversification of marine organisms is unclear. Diversity is generated when a single interbreeding population diverges into multiple populations, and it is maintained when differences arise between those populations such that they can no longer interbreed. Yet documented instances of hybridization and gene flow between distinct populations complicate this binary model in which populations either can or cannot interbreed. Hybridization followed by gene flow was once considered rare, but recent investigations have revealed that gene flow upon secondary contact is a common feature of the evolutionary history in many lineages (Edelman and Mallet 2021), including lineages that demonstrate no evidence of contemporary hybridization (Payseur and Rieseberg 2016, Suvorov et al. 2021, Taylor and Larson 2019). Increasing evidence indicates that some barriers to gene flow, whether phenotypic, genotypic, or environmental, are dynamic and change over space and time (Colella et al. 2018, Mandeville et al. 2017, Schumer et al. 2017, Taylor et al. 2014, Zieliński et al. 2018). These advances in our understanding have been facilitated by the increasing availability of high-throughput molecular-sequencing technologies (Campbell et al. 2018).

Physical barriers to dispersal are commonly associated with restrictions in gene flow and subsequent population differentiation and speciation (e.g. Dolby et al. 2019, Dong et al. 2020, Nevado et al. 2018, Spellman et al. 2007). Yet physical barriers to

dispersal are not always absolute barriers to gene flow, and some evidence suggests that physical barriers may play a reduced role in speciation in marine environments compared to terrestrial ones (Costa et al. 2021, Faria et al. 2021, Kess et al. 2021, Laakkonen et al. 2021, Prada et al. 2021, Ravinet et al. 2021, Simon et al. 2021). The possibility of speciation in the absence of absolute physical barriers to dispersal has received much less attention (Friesen 2015), but research into patterns of gene flow between and within marine species can help to fill this gap.

Seabirds are highly mobile animals, and species are often spread across vast distances with few to no physical barriers to dispersal. Seabird sister species often overlap in distribution with no land barriers to gene flow and, in many of these instances, gene flow is restricted instead by ecological or behavioral differences or even specialization to different ocean regimes (Friesen 2015), as is the case with blue-footed (*Sula nebouxii*) and Peruvian boobies (*S. variegata*; Duffy 1987, Taylor et al. 2011a, Taylor et al. 2011b, Zavalaga et al. 2008, Zavalaga et al. 2010). The mechanisms by which seabird populations differentiate and ultimately speciate in the absence of physical barriers to dispersal are uniquely interesting to our understanding of evolutionary processes, as speciation patterns determined by nonphysical barriers might be more apparent in these circumstances (Friesen 2015). Hybridization, though common in birds (Price 2008), has only been documented in several instances for tropical and subtropical seabirds (Brown et al. 2015), and the extent to which this hybridization has been followed by introgression is under-characterized (but see Taylor et al. 2012a, Taylor et al. 2013). Introgression has been documented based on genetic data analysis in quite a few more instances involving temperate, subpolar, and polar breeding seabird species, including

murres (*Uria spp*., Taylor et al. 2012b), petrels (*Macronectes giganteus* and *M. halli*, Brown et al. 2015), gulls (*Laridae* spp. Sternkopf et al. 2010, Stonsthagen et al. 2016, Mischler et al. 2018), and shearwaters (*Puffinus* spp. Austin 2004).

Previous investigations into gene flow between and within seabird species have largely depended on mitochondrial sequence data and microsatellites, and, to some extent, nuclear intron sequence data (Friesen 2015). In contrast, investigation of genome-wide patterns of gene flow and introgression in tropical and subtropical seabirds can provide critical insights into mechanisms of speciation, and several recent whole-genome sequencing (WGS) studies have already advanced our understanding of seabird speciation (Mikkelsen et al. 2022, Pan et al. 2020, Rexer-Huber et al. 2019, Tigano et al. 2017, Tigano et al. 2018, Vianna et al. 2020). With WGS analyses, we can investigate questions of speciation with higher resolution than ever before.

In tropical and subtropical seabirds, the presence of land — primarily the Isthmus of Panama and the African continent (Friesen 2015) — is a major barrier to gene flow (Lombal et al. 2020). Yet growing evidence indicates that seabird individuals will travel across land barriers (Booth Jones et al. 2017), and that substantial variation exists between and within species in patterns of philopatry (Coulson 2016). The importance of land barriers to tropical seabird speciation may thus be overstated and should be tested with genome-wide sequence data.

Restricted gene flow has been found between colonies that do not span a land or ice barrier but that are spread across different ocean regimes, and sister species are often found in sympatry (Friesen 2015). This pattern is particularly prevalent in the eastern Pacific Ocean. For example, this region is home to three sister pairs of storm-petrel

species (Family *Hydrobatinae*; Wallace et al. 2017), and genetically distinct populations of brown pelicans (*Pelecanus occidentalis*; Taylor et al. 2018), magnificent frigatebirds (*Fregata magnificens*; Gonzalez-Jaramillo and Rocha-Olivares 2011; Hailer et al. 2011) and great frigatebirds (*F. minor*; Dearborn et al. 2003; Levin et al. 2012), as well as species endemic to the Galápagos Islands and surrounding area, including flightless cormorants (*Phalacrocorax harrisi*; Duffie et al. 2009), Nazca boobies (*Sula granti*; Levin et al. 2012), waved albatrosses (*Phoebastria irrorata*; Huyvaert and Parker 2006), Galápagos penguins (*Spheniscus mendiculus*; Nims et al. 2008), and Galápagos petrels (*Pterodroma phaeopygia*; Friesen et al. 2006; Welch et al. 2011). The speciation processes that underlie this unique pattern in the eastern Pacific Ocean are largely unknown. For example, we do not know if gene flow occurs between sympatric species in the eastern Pacific Ocean, and investigations into the presence or absence of barriers to gene flow in this region would provide an important foundation for future studies of speciation in this ecologically unique region.

The boobies (genus *Sula,* family *Sulidae*) constitute a genus of six tropical seabird species. Instances of interspecific courting or nesting, hybrid offspring, or a combination of the three have been documented for several species' pairs. Three booby species are pantropically distributed: red-footed boobies (*S. sula*), brown boobies (*S. leucogaster*), and masked boobies (*S. dactylatra*). The other three species are endemic to the eastern Pacific Ocean: Nazca boobies, blue-footed boobies, and Peruvian boobies (Figure 1). Two studies, one using cytochrome-b sequence data and a subsequent study using five nuclear introns plus mtDNA, supported a phylogenetic hypothesis with blue-footed and Peruvian boobies sister, masked and Nazca boobies sister; these four species forming a

sister clade to brown boobies; and red-footed boobies as the most diverged lineage (Friesen and Anderson 1997, Patterson et al. 2011).

Studies using a mix of mitochondrial, microsatellite, and multi-locus nuclear sequence data have identified three major populations within each of the pantropical species: one each in the Pacific, Indian, and Atlantic Oceans (Morris-Pocock et al. 2010, Morris-Pocock et al. 2011, Steeves et al. 2003, Steeves et al. 2005a, Steeves et al. 2005b). The Pacific and Indian Ocean populations of the masked booby are less distinct from each other than are those of red-footed and brown boobies. Thus, the Isthmus of Panama and the continent of Africa present physical barriers to gene flow in masked boobies, but the barrier restricting gene flow between the Pacific and Indian Oceans is less clear. For red-footed boobies, gene flow between the Pacific and Indian Oceans was apparently restricted by the Sunda and Sahul shelves (Morris-Pocock et al. 2010), which are a southward extension of the Asian continental shelf and a northward extension of the Australian continental shelf, respectively, and which would have formed an intermittent solid land mass between the Pacific and Indian Oceans in the mid-Pleistocene. However, masked boobies expanded throughout the Pacific and Indian Oceans about 180,000 years ago, when the Torres Strait never fully closed, and so land barriers were unlikely to fully restrict gene flow between these oceans (Steeves et al. 2005b). One study observed blue-footed boobies flying over land in the Galápagos Islands (Anchundia et al. 2017), but no quantitative study has been performed of any booby species flying over a continental landmass. Thus, land barriers have shaped population structure in these species, but little to no evidence exists to indicate if these land barriers have been breached by some of the pantropical species since initial population divergence.

Evidence of interspecific courting and hybridization has been documented between multiple species of boobies. Hybrids of two sympatric pairs of booby species in the eastern Pacific have been observed and confirmed with genetic data (blue-footed and Peruvian, Taylor et al. 2010b, 2012a; and blue-footed and brown, Taylor et al. 2013). A putative hybrid between brown and masked boobies was photographed on Ascension Island in 2002 (Richard White, pers. comm.). A blue-footed booby was observed courting a Nazca booby (Figueroa and Stucchi 2008), and Nazca booby foot color varies extensively in the olive–purple-green range, but can appear blue to the human eye (D. Anderson pers. obs.), but no evidence of hybridization exists. Masked and Nazca boobies also overlap in distribution in the eastern Pacific and a mixed species pair has been observed on San Benedicto (T. Steeves pers. obs.), but no hybrids have been observed, although hybrids could be indistinguishable to the human eye due to the morphological similarity of these species.

In this study, we analyzed 30 low coverage (approximately 5x) whole-genome sequences from all six of the tropical boobies in the genus *Sula* with two objectives:

(1) Test for evidence of introgression between five pairs of sympatric booby species in the eastern Pacific Ocean.

(2) Test for evidence of gene flow across land barriers in the three pantropical booby seabird species.

Our results help clarify the role of physical barriers, introgression, and gene flow in generating contemporary patterns of diversity in this charismatic group of seabirds and have implications for our understanding of the speciation patterns of broadly distributed marine species.

Methods

We extracted DNA from blood samples from 34 birds that were sampled previously for population genetic analyses (Figure 1; Friesen et al. 2002, Morris-Pocock et al. 2011, 2016, Steeves et al. 2003, 2005a, 2005b, Taylor et al. 2010a, 2011a, 2011b) and were archived at Queens University. We extracted the DNA at the University of Colorado using a Qiagen DNeasy Blood & Tissue kit and a Thermo Scientific GeneJET Genomic DNA Purification Kit. We measured DNA concentrations on a Thermofisher Qubit 3.0, and we used a Zymo Research DNA Clean & Concentrator kit to concentrate samples with low DNA concentrations. Libraries were prepared using the Nextera XT V2 library kit and were sequenced at the Genomics and Microarray Core at the University of Colorado Anschutz Medical Campus with an Illumina NovaSEQ 6000 with a Paired End 150 cycle 2x150. Raw sequence data are publicly available for download through the Sequence Read Archive (BioProject accession: PRJNA836623).

All bioinformatic scripts with specific settings can be found on GitHub (https://github.com/dannyjackson/sula). We trimmed and analyzed for quality raw sequence fasta files using Trimmomatic (Bolger et al. 2014) and FastQC (Andrews 2010). We aligned the trimmed files using bwa mem (Li 2013) to a flightless cormorant genome (NCBI ID 55342, Assembly GCA_002173475.1 Pharrisi_ref_V1, 2,651

scaffolds, 31,595 contigs, N50 = 100,243 L50= 3,591; Burga et al. 2017) and we sorted and indexed the bam files using samtools (Li et al. 2009) and picard-tools ("Picard Toolkit" 2019). We selected this genome as it is the highest quality genome from the closest related outgroup clade. We confirmed that our analyses were not biased by the use of a too-distant genome (Prasad et al. 2022) by repeating several interspecific analyses with fastq files aligned to a masked booby genome assembled by the Bird 10,000 Genomes (b10k) Project (sampled in Louisiana; Feng et al. 2020). We found similar patterns, and so to avoid redundancy we only present the results based on the flightless cormorant, as an in-group genome might skew some of the intraspecific analyses. We called and filtered variant SNPs using BCFtools (Narasimhan et al. 2016). We excluded four individuals with higher than 0.25 frequency of missing SNPs from analyses (brown booby 4, blue-footed booby 6, Nazca booby 1, Peruvian booby 2). We extracted DNA from a masked booby from the eastern Pacific Ocean (masked booby 3), but sequencing was unsuccessful and this sample was excluded from this analysis. Remaining samples span much of the range of each pantropical booby species (six red-footed booby samples, four brown booby samples, and five masked booby samples), excluding red-footed boobies from the central and eastern Atlantic as well as the far western Pacific, brown boobies from the Indian Ocean and western Pacific Ocean, and masked boobies from the eastern Pacific Ocean (Figure 1). Maps of full species distributions can be found in the supplement (Figure S12). These samples also span much of the range of the Nazca booby, the blue-footed booby, and the Peruvian booby, excluding the southernmost extent of the Peruvian booby breeding range.

First, we confirmed that the sequences clustered in the expected species and populations (based on previous population genetic data and phylogenetic analyses) using a Randomized Accelerated Maximum Likelihood (RAxML; Stamakis 2014) analysis and principal component analyses (PCA). We converted the filtered VCF to a Phylip file (Ortiz 2019) for RAxML analyses, and we ran RAxML using a Felsenstein ascertainment bias correction to account for the absence of non-variant sites, under the GTRCAT model with 1000 random seeded bootstrap replicates. We allowed RAxML to halt bootstrapping automatically using the autoMRE criterion. We visualized the tree in FigTree (Rambaut 2018), and we present the best tree with bootstrap support values. We conducted these in R using gdsfmt and SNPRelate (Zheng et al. 2012). We performed each PCA with a subset of all SNPs that we pruned for linkage disequilibrium (ld) using an ld threshold of 0.2. We performed PCAs in the following clades: 1. all samples; 2. masked, Nazca, blue-footed, and Peruvian booby samples; 3. masked and Nazca booby samples; 4. blue-footed and Peruvian booby samples; 5. red-footed booby samples; 6. brown booby samples; 7. masked booby samples; 8. Nazca booby samples; 9. blue-footed booby samples; 10. Peruvian booby samples. Then, we tested for patterns of shared genetic variation between individuals across populations using four-taxon ABBA-BABA statistics (Martin et al. 2015) and phylogenetic network analysis in Phylonet (Than et al. 2008, Wen et al. 2018).

F-Statistics

F_{ST} values were calculated between each of the sister species (masked versus Nazca, blue-footed versus Peruvian), between the two populations of masked boobies and between the two populations of brown boobies (Atlantic and Caribbean versus Indo-

Pacific; Weir and Cockerham 1984). Calculations were performed in VCFtools (Danecek et al. 2011).

D-statistics

D-statistic tests are a powerful method to identify histories of introgression between populations (Martin et al. 2015), and they can test as few as one individual per tree tip (Hahn 2019). They have most often been applied at the species level because taxa must exhibit population isolation with reliable phylogenetic relationships to meet the assumptions of this test. Given that boobies breed on isolated island colonies and almost all have been shown to exhibit population genetic structure among those colonies, these populations meet the assumptions of the D-statistic. We never split Peruvian booby genomes into more than one population for our tests because this species demonstrates panmixia across its range (Taylor et al. 2011b).

D-statistic tests are most commonly performed using four populations with the following relationships: (((P1,P2),P3),O). If populations diverge in the absence of gene flow, we would observe many sites that follow a (((B, B), A),A), where A is the ancestral genotype and B is a derived genotype. We would also observe some sites that follow a (((A, B), B), A) or a (((B, A), B), A) pattern, due to either incomplete lineage sorting or gene flow. We expect these latter two patterns to occur at similar frequencies except in the presence of gene flow between P2 and P3 at a time point more recent than the split between P1 and P2, which would result in a higher frequency of ABBA sites as compared to BABA sites. This pattern can also occur in the absence of introgression due to incomplete lineage sorting resulting from ancestral population structure; therefore,

significant D-statistics can indicate either introgression or ancestral population structure (Martin et al. 2015). In many cases, one or the other can be inferred from the natural histories of the studied organisms.

The D-statistic does not quantify the amount of shared variation between P2 and P3 as compared to the shared variation between P1 and P3, and so only the signifier of the D-statistic is relevant to the hypothesis test: if the D statistic is positive with a p-value lower than our acceptable alpha, we reject the null hypothesis of no introgression. In other words, a high D-statistic does not necessarily indicate that a greater proportion of the genome has introgressed compared to a lower, but still significant, D-statistic (Martin et al. 2015).

We performed five tests of interspecies introgression and nine tests of intraspecies introgression (Table 1, Table S1). Our null hypothesis for each interspecies test was that P2 and P3 do not exhibit more shared genetic variation than P1 and P3, which is a one-tailed test. Our null hypothesis for each intraspecific test was that neither P2 nor P1 exhibits more shared genetic variation with P3, which is a two-tailed test. We set our acceptable alpha to 0.01, or a Z score of 2.34 for interspecies tests and of |2.58| for intraspecies tests. We report all intraspecies test results in a format such that the D and Z are positive, as these are exploratory two-tailed tests, but report interspecies test results in the pre-defined four-taxon tree format, as these tests were informed by evidence of hybridization.

All D-statistics and their accompanying standard deviations and Z scores were calculated in R, using a geno file that was converted from the VCF table in python using scripts and tutorials made available by Simon Martin (Martin et al. 2015). These scripts

use site frequencies rather than absolute A or B values at a site, which would result in a significant reduction in sites available for the calculations.

We tested five hypotheses for interspecific introgression (Table 1). Only alternative hypotheses are listed. Null hypotheses are always that neither P1 nor P2 shares more genetic variation with P3 in the four-taxon tree (((P1, P2), P3) P4). Because of the results of the initial analysis for H2, we tested several follow-up hypotheses about the relationships between brown boobies and the four-taxon clade (blue-footed, Peruvian, Nazca, and masked boobies). The RAxML analysis does not support a (((P1,P2),P3)O) tree for all of the samples from the species in H3, but the PCA analyses do. And for the specific samples used in the H3 analysis, both RAxML and PCA support this relationship.

Intraspecific hypothesis tests were performed on nine sets of within-species genomes that fit a (((P1,P2),P3),O) phylogenetic pattern for the three pantropical booby species. These tests were designed to test for patterns of gene flow across land barriers, and we excluded tests that relied on nodes with low bootstrap support (i.e. any involving red-footed booby 4).

Phylonet

We constructed a phylogenetic network at the species level with Phylonet v.3.8.2 (Than et al. 2008, Wen et al. 2018) following methods from Mikkelsen and Weir (2022) to further test for patterns of ancestral introgression. We first used Plink to split the VCF into individual chromosomes, and then we used shapeit to phase genotypes into haplotypes using the read-aware option that uses phase informative reads (PIRs)

extracted from bam files (Delaneau et al. 2008). We filtered phased scaffolds to exclude any scaffolds where any individual had more than 25% missing data, and masked fastas using bedtools maskfasta (Quinlan and Hall 2010, Quinlan 2014). Finally, we split the fastas into 5,000 bp haplotypes that were each 10,000 bp apart for input into Phylonet. We used the InferNetwork_MPL option to construct phylogenetic networks with 1–4 reticulations, restricting the tree with the taxa map option to only model relationships between species. We performed three repetitions with each possible number of reticulations to obtain 12 possible models. Finally, we selected the best model by computing AIC, AICc, and BIC scores for each model, and we compared the top five models from each repetition for a total of 60 tested models. All three metrics selected the same model, and we present the model with the lowest score of all three.

Results

DNA Extraction, sequence alignment and filtering

We obtained 1.6×10^9 raw reads across 34 individuals, and we retained 1.2×10^9 after trimming, with a mean of 36,050,833 reads per individual and a mean read depth of 4.57 per individual. After filtering, the variant call file contained 9,224,458 total variants.

We identified 13,131 fixed sites between masked and Nazca boobies (genome-wide Weir and weighted $F_{ST} = 0.14$), and 6343 fixed sites between blue-footed and Peruvian boobies (Weir and Cockerham weighted F_{ST} of 0.14). Weir and Cockerham weighted F_{ST} between the Atlantic Ocean and Caribbean Sea masked booby population and the Indian Ocean and Pacific Ocean population was 0.041, and between the same population split of brown boobies was 0.067. Fixed sites are not reported between

conspecific populations due to small sample size for each population, and F_{ST} values are not reported between red-footed booby populations because the Indian Ocean and Pacific Ocean samples did not form a monophyletic clade in our analysis.

Phylogenetic Relationships Among Taxa

The RAxML phylogeny matched species-level relationships identified in previous work (Figure 2; Friesen and Anderson 1997, Patterson et al. 2011). Red-footed boobies diverged first, followed by brown boobies. A four-taxon clade is sister to brown boobies, with two pairs of sister species within it: blue-footed with Peruvian boobies and masked with Nazca boobies. Brown and masked boobies each split into an Atlantic Ocean/Caribbean Sea clade and a Pacific Ocean/Indian Ocean clade. Patterns in the red-footed booby clade are less immediately interpretable due to a node with low bootstrap support, but the analysis revealed a clade of Pacific Ocean samples positioned within samples from the Caribbean Sea and Indian Ocean. Peruvian, blue-footed, and Nazca booby samples showed no within-species phylogeographic patterns. These patterns all align with phylogeographic findings from previous studies (Friesen et al. 2002, Morris-Pocock et al. 2011, 2016, Steeves et al. 2003, 2005a, 2005b, Taylor et al. 2011a, 2011b). Interpretations of intraspecific patterns should acknowledge that we did not sample every colony within each species range, and that a future genomic analysis with broad sampling could reveal more refined patterns.

All interspecies PCAs revealed clusters similar to the RAxML analysis (Figures S1, S2, S3, and S4). Intraspecific analyses identified patterns consistent with those expected based on geography and previous publications (Morris-Pocock et al. 2010,

Morris-Pocock et al. 2011, Steeves et al. 2003, Steeves et al. 2005a, Steeves et al. 2005b, Taylor et al. 2011a, Taylor et al. 2011b). In the analysis of all species, PC1 and PC2 explained relatively low percentages of variation (6.54% and 5.63% respectively). These low numbers reflect the setup of the PCAs because genomic samples of multiple individuals across four species cluster together.

Intraspecies PCAs also revealed similar clusters of population assignment to the RAxML analyses (Figures S1-S4). For red-footed boobies (Figure S5), Principal Component 1 (PC1) separated the Caribbean Sea and Indian Ocean samples from the Pacific Ocean samples (6 samples, PC1 = 20.91% variation). In brown boobies (Figure S6), PC1 split the samples between the Pacific Ocean, and the Caribbean Sea and Atlantic Ocean (4 samples, PC1 = 38.30% variation). In masked boobies (Figure S7), PC1 again split the samples based on assignment to either the Pacific and Indian Oceans or the Caribbean Sea and Atlantic Ocean (PC1 = 29.73% variation). No patterns were discernible in the PCA of the Nazca booby samples (Figure S8). PC1 separated the two southernmost Peruvian booby samples from all other samples (Figure S9, 5 samples, PC1 = 25.49% variation). PC1 separated the northernmost blue-footed booby sample from all other samples (Figure S10, 5 samples, 25.86% variation).

Patterns of Introgression between species

Three of our five tests for interspecific introgression were significant (Figure 2, Table 1). Test 1 indicated that Nazca booby genomes show evidence of introgression with masked booby genomes from the Pacific Ocean since the time that Pacific Ocean masked boobies diverged from masked boobies in the Caribbean Sea and Atlantic Ocean.

Test 2a was significantly negative, providing no evidence for a history of introgression between blue-footed and brown boobies in the Pacific Ocean, but instead supporting historical introgression between blue-footed boobies and brown boobies in the Atlantic Ocean and Caribbean Sea. Test 3 indicated that Peruvian boobies show evidence of introgression with blue-footed boobies from the coast of northern Peru since the time that blue-footed boobies on that island diverged from blue-footed boobies from the Gulf of California. Test 4 indicated that Nazca boobies show evidence of introgression with blue-footed boobies since the time that blue-footed boobies diverged from Peruvian boobies. Test 5 did not find evidence of introgression between brown boobies from the Atlantic with masked boobies from the Caribbean Sea and the Atlantic Ocean.

To follow up on the finding from Test 2a that blue-footed boobies show evidence of introgression with brown boobies east of the Isthmus of Panama, rather than with the sympatric eastern Pacific population of brown boobies, we tested several additional four-taxon sets. Test 2b also tested for introgression between blue-footed and brown boobies but with different population splits and could be more sensitive to very recent introgression than Test 2a; however, it also found no evidence for introgression. Tests 2c, d, and e all indicate that brown boobies in the Atlantic Ocean and Caribbean Sea show evidence of introgression with each of the three species that form a clade with blue-footed boobies (Peruvian, masked, Nazca). Test 2f indicated that brown boobies in the Pacific Ocean show evidence of introgression with Peruvian boobies since the time that Peruvian boobies diverged from blue-footed boobies.

Intraspecific D-statistic analyses most often supported a model in which gene flow has occurred across all examined land and oceanic barriers in all three species,

except we found no evidence for gene flow across Indonesia in red-footed boobies. Tests 5a and b (Figure 3, Table S1) indicated that red-footed boobies in the Indian Ocean share more genetic variation with each other than with samples from the Pacific Ocean, which supports the conclusion that population structure in this clade is defined by ocean basin. Tests 5c and d all indicate that samples in the Indian Ocean share more genetic variation with samples from the eastern Pacific Ocean than with samples from the central Pacific. Test 6a found that brown boobies to the east of the Isthmus of Panama share more genetic variation with the more proximate sample from the Gulf of California than they do with the more distant sample from the central Pacific Ocean (Figure 3, Table S1). Test 6b found that the samples to the west of the isthmus share more genetic variation with the more proximate sample in the Caribbean Sea than with the more distant sample from the Atlantic Ocean. Test 7a indicated that masked booby samples from the Atlantic Ocean share more genetic variation with a more proximate sample from the western Indian Ocean than with a more distant sample from the western Pacific Ocean (Figure 3, Table S1). Test 7b indicated that samples from the Atlantic Ocean and Caribbean Sea share more genetic variation with the most proximate sample from across the Isthmus of Panama than with the most proximate sample from across Africa. Test 7c indicated that all samples outside of the Caribbean Sea and Atlantic Ocean clade share more genetic variation with the sample from the Caribbean Sea than with the sample from the Atlantic Ocean.

AIC, AICc, and BIC all selected the same model (Table S2, S3, and S4). Phylonet recovered the same species relationships as our RAxML model, with blue-footed and Peruvian boobies sister, masked and Nazca boobies sister, the two clades sister to each

other, and brown boobies sister to that four taxon clade (Figure S11). We set red-footed boobies as the outgroup. The model uncovered four reticulations, (light blue lines on Figure S11). It found introgression between the ancestral population of Peruvian and blue-footed boobies and four other lineages (in order from most recent to most ancient events): #1 a ghost lineage that is sister to blue-footed boobies, #2 Peruvian boobies, #3 the ancestor of masked and Nazca, and #4 the ancestor of the four-taxon clade (masked, Nazca, blue-footed, and Peruvian boobies), These suggest divergence in the presence of gene flow within this four taxon clade.

Discussion

We found evidence for introgression between multiple booby species pairs and across land barriers within all pantropical booby species. This supports a model of speciation with gene flow at multiple stages in the divergence process and implies that further investigations into speciation in marine organisms would greatly expand our understanding of the dynamic processes underlying the generation of biodiversity.

We found evidence from whole-genome sequences of introgression between three booby species pairs, but not between two pairs of species with documented occurrences of contemporary hybrid offspring. We also found some indication of ancient hybridization between brown boobies and the ancestor of the blue-footed, Peruvian, masked, and Nazca booby clade. We found unexpected evidence for introgression between Peruvian boobies and brown boobies, which do not overlap in their contemporary breeding distributions, but brown boobies have been regularly observed visiting Peruvian islands and resting close to Peruvian boobies (Valverde 2007, C.

Zavalaga pers. obs.). Finally, as predicted, we found evidence of historical gene flow across the Isthmus of Panama in all three pantropical species and between the Atlantic and Indian Oceans in red-footed and masked boobies, with additional support for population structure by ocean basin for masked and brown boobies.

Evidence for interspecific gene flow between three booby species pairs

The ABBA BABA tests and the Phylonet analysis uncovered different patterns of introgression, although each indicates widespread introgression between closely related booby species in the eastern Pacific Ocean. While the phylogenetic network suggests some interesting ancestral patterns of gene flow, with suggestions of ghost lineages and frequent introgression events between populations throughout the process of speciation, we focus our discussion on the specific results of the ABBA BABA tests. These tests were designed based on field observations, natural histories, and geographic overlap and are hypothesis driven. While the speculative model of Phylonet highlights interesting avenues for future research, these patterns are neither supported nor refuted by field data, and they probably cannot be directly tested given the rarity of seabird fossils and the resulting lack of prehistorical data on seabird distributions.

No land barrier currently restricts gene flow between the three species pairs that demonstrated introgression in our analyses (blue-footed and Peruvian boobies, masked and Nazca boobies, and, unexpectedly, blue-footed and Nazca boobies). Our whole-genome investigation supports previous microsatellite evidence for introgression between blue-footed and Peruvian boobies (Taylor et al. 2012a). Investigations into the interactions between introgressed regions of the genome and the selective environments

of these respective species are ongoing and will be necessary to better characterize speciation in tropical and subtropical boobies. We also found evidence that gene flow has occurred between masked and Nazca boobies since the divergence of masked booby populations on either side of the Isthmus of Panama. The Atlantic Ocean and Pacific Ocean populations were dated using mitochondrial sequence data to have split about 0.2–0.3 mya, the Indian Ocean and Pacific Ocean populations of masked boobies were dated to have expanded about 180,000 years ago (95% CI 179,000–239,000 years ago, Steeves et al. 2005a), and masked and Nazca boobies were dated to have split 0.8 [0.1–1.7] mya (Patterson et al. 2011). Nazca and masked boobies are nearly indistinguishable to the casual observer, but the two species differ in all measured characteristics including bill color, breeding habitat, extent of sexual dimorphism, and other aspects of their morphology and ecology (Pitman and Jehl 1998, Van Oordt et al. 2018). Investigations into the role of morphological and ecological differences in maintaining species boundaries would provide interesting context for the process of speciation in this clade.

The finding that introgression has occurred between blue-footed and Nazca boobies is surprising given their relatively deep divergence (dated to 1.6 [0.7–2.8] mya, Patterson et al. 2011), and given that no hybrid individuals between these species have been documented. Blue-footed and Nazca boobies in the Galápagos frequently breed in proximity (Townsend et al. 2002) and, if contemporary hybridization between these species were anything but a rare occurrence, it would probably have been documented. It is possible that introgression between Nazca and blue-footed boobies occurred shortly after the split between blue-footed and Peruvian boobies, and that selection against hybrids reduced or eliminated all contemporary examples of this phenomenon. However,

Nazca boobies with foot color phenotypes similar to blue-footed boobies have been observed (D. Anderson, pers. obs.). It is interesting that population genetic structure is found across ocean regimes with no land barriers in some booby species, and that sister species co-occur with no physical barrier to gene flow, and yet none of these species pairs — neither blue-footed and Peruvian, masked and Nazca, nor blue-footed and Nazca — show evidence of converging in our phylogenetic analyses despite some gene flow. Our findings imply that introgression can be an integral mechanism to the speciation processes in widespread marine organisms, and that selection can drive population differentiation and, potentially, speciation.

We did not find evidence for introgression between blue-footed and brown boobies or between masked and brown boobies, despite documented instances of hybridization (Taylor et al. 2013). Of all four tests for interspecific introgression, these taxa are the most divergent (dated to 2.7 [1.2–4.3] mya, Patterson et al. 2011). The absence of evidence for introgression between blue-footed and brown boobies or between masked and brown boobies suggests that either genomic incompatibilities or behavioral isolation of hybrids maintain reproductive isolation despite occasional hybridization. In our tests for introgression between blue-footed and brown boobies, we also found consistent evidence that each of the four booby species (blue-footed, Peruvian, masked, and Nazca) shares more genetic variation with brown boobies in the Atlantic Ocean/Caribbean Sea than they do with brown boobies in the eastern Pacific Ocean. Our Phylonet analysis did not predict any introgression events between brown boobies and any other lineage. However, this model predicts the most likely introgression events and, given the high number of introgression events that we identified within the four-taxon

clade, Phylonet may not be able to detect more ancestral introgression events in the presence of recent introgression without permitting an unreasonably high number of reticulation events in the model. We might observe these patterns if the population structure observed in brown boobies predated the split between brown boobies and the blue-footed, Peruvian, masked, and Nazca clade, and if that four-taxon clade descended from the ancestral population of the contemporary Atlantic/Caribbean brown booby population. A multilocus population genetic analysis of brown boobies estimated that eastern Pacific Ocean brown boobies diverged from all other populations around 1 mya and that brown boobies diversified in the absence of gene flow (Morris-Pocock et al. 2011). A multilocus phylogeny of the Sulidae estimated the origin of the masked, Nazca, blue-footed, and Peruvian booby clade to be 2.1 mya [0.1–5.1], relaxed clock, or 1.6 mya [0.7–2.8] strict clock (Patterson et al. 2011). Gene flow after the initial divergence event would disrupt both estimations, and if brown boobies exhibited more gene flow between their populations than they did with other sulid species throughout this period, the true date of brown booby population divergence would be more than the estimate of ~1 mya. Earlier analyses of brown booby mitochondrial sequence data put divergence times between the populations on either side of the Isthmus of Panama at 0.38–1.5 mya (Steeves et al. 2003) and 0.20–0.28 mya (Morris-Pocock et al. 2010). The evidence that hybridization and introgression occur between sister sulid species and even between blue-footed and Nazca boobies, but not between more diverged but hybridizing lineages, suggests that species boundaries are maintained in the presence of gene flow.

We also uncovered unexpected evidence of introgression between Peruvian boobies and brown boobies (Test 2f), and this finding should be interpreted with caution.

The four-taxon ABBA-BABA test was designed specifically to test for introgression between sympatric populations of diverged species based on predefined hypotheses, but these species are not currently sympatric. To our knowledge, little to no fossil evidence indicates that they were ever sympatric, largely because documented fossils on seabird breeding colonies are relatively rare. For instance, no fossil specimen of Peruvian boobies or brown boobies exist on the Global Biodiversity Information Facility (GBIF) database as of July 12, 2023. No models of the ancestral ranges of these species under historic climates have been constructed (but see Quillfeldt and Masello 2013, Cursach et al. 2019). However, a record of a brown booby on islands in Perú alongside Peruvian boobies depicts an individual with a brown head, which could either be a female from the Pacific Ocean or an individual of either sex from the Atlantic Ocean (Valverde 2007). A recent model of global sea surface temperatures over the past 24,000 years found warmer surface air temperatures over the Southern Ocean at between 9 kya and 2 kya compared to the most recent 2,000 year period (Osman et al. 2021). This could have altered the distribution of both species and facilitated hybridization between Peruvian boobies and brown boobies either in the Pacific Ocean or around Tierra del Fuego, either of which could drive this pattern. Given the documented examples of contemporary hybridization between other booby species, and evidence that we provide here for more recent introgression events, introgression may have shaped the deep histories of this clade in ways that current analytical frameworks cannot fully untangle.

Gene flow across land barriers in red-footed boobies

We found evidence that gene flow has occurred in red-footed boobies across most existing land barriers but is reduced or nonexistent across a historical land barrier that no longer exists. This implies that land barriers alone do not facilitate population differentiation and speciation, and that additional factors must be at play. The evidence of no gene flow within red-footed boobies across the Indonesian Archipelago is perhaps the most interesting finding of these intraspecific analyses, as this land barrier no longer exists. Additionally, previous models developed from nuclear introns and microsatellites using Bayesian population assignment supported a history of some low level of gene flow between populations in the Indian Ocean and the Pacific Ocean (Morris-Pocock et al. 2016), although all studies indicate a deep split between Indian Ocean and Pacific Ocean populations. Additional whole-genome studies of red-footed boobies across Indonesia could give insights into the mechanisms that reduce gene flow in this region. No tests could directly investigate gene flow across the Isthmus of Panama and across Africa, as the sample from the Caribbean was placed with uncertainty in the phylogeny, but Tests 5c and 5d indicated that gene flow had occurred across both.

Some evidence of gene flow across the Isthmus of Panama in brown boobies

We found evidence for gene flow across the Isthmus of Panama in brown boobies. Previous studies with mitochondrial DNA found no evidence of gene flow out of the Caribbean Sea for brown boobies (Morris-Pocock et al. 2010). This pattern could have resulted from ancestral population structure rather than more recent gene flow over the land barrier. For example, if the ancestral populations on either side of the American continents exchanged genes before the formation of the Isthmus of Panama, and that gene

flow was subsequently restricted between colonies in the Caribbean and Atlantic, we might observe a similar pattern as in these tests. However, a multilocus analysis of brown boobies found that the eastern Pacific populations were the most diverged from other brown boobies (Morris-Pocock et al. 2011). Additional genomic work including samples from the Indian Ocean and western Pacific Ocean are needed to better characterize gene flow in this species.

Gene flow across all land barriers in the masked booby

We found evidence for gene flow across all land barriers in the masked booby. One finding contradicts previous work in this species: samples between Africa and the Americas showed more shared genetic variation with the sample from the central Pacific Ocean than with the sample from the western Indian Ocean, indicating that gene flow across the Isthmus of Panama was greater than gene flow around Africa. However, Steeves et al. (2005) discovered a mitochondrial haplotype in the Caribbean Sea that clustered with samples from the Indian Ocean, indicating that gene flow around Africa had occurred, probably during a period when warm water pulsed around the tip of Africa approximately 130,000 years ago. We cannot distinguish patterns of gene flow across the Isthmus of Panama before the land barrier formed from those occurring after, and so the observed patterns of shared genetic variation could be a result of either or both. Regardless, this evidence reveals that gene flow has occurred at some point between Pacific Ocean populations of masked boobies and populations in the Caribbean Sea and Atlantic Ocean across the Americas.

Summary and broader implications of our findings

What drives speciation in widespread marine organisms? In the two most closely related sister species pairs, masked and Nazca boobies, and blue-footed and Peruvian boobies, we detected evidence of introgression across species boundaries. The species in the first pair are phenotypically very similar, distinguishable to most observers by only slight variations in bill coloration (Nazca boobies have a slightly more orange or pink hue than masked boobies, Pitman and Jehl 1998), while the second species pair is quite dissimilar, particularly in foot coloration, a trait that has been shown to be influential in mate choice in blue-footed boobies (Torres and Velando 2003, 2005), and in eye color and plumage. We also detect introgression between Nazca and blue-footed boobies, a pair of species that is quite divergent both genetically and phenotypically. And we found tentative evidence for historical introgression between brown boobies and the ancestor to the clade including blue-footed, Peruvian, masked, and Nazca boobies.

How long do sulid populations need to maintain reproductive isolation before hybridization and backcrossing no longer occur? The evidence of recent introgression between Nazca boobies and masked booby populations in the Indo-Pacific is difficult to reconcile with the patterns of widespread gene flow in the masked booby. It supports a theory of parapatric speciation due to strong selection around the Galápagos Islands. Given the lack of any absolute barriers to gene flow in this species, how did the ancestral population of the Nazca booby differentiate enough to maintain reproductive isolation in geographic proximity to masked booby colonies? Similar questions arise from the evidence for introgression between blue-footed and Peruvian boobies, as well as blue-footed and Nazca boobies. And yet our data failed to support a pattern of introgression

between blue-footed and brown boobies, which have been observed hybridizing and producing viable offspring. We might infer that introgression cannot occur between these diverged species, although the mechanisms preventing introgression (e.g., hybrid sterility or behavioral selection against hybrids) remain to be explored.

No evidence of recent hybridization between blue-footed and Nazca boobies has been reported, so the finding of introgression between the two raises interesting questions for further research. Have hybridization rates between these two species declined over evolutionary time because of behavioral reinforcement? These findings call for further investigations into behavioral and genomic factors that generate and maintain population differentiation and species boundaries in this clade and in marine organisms with few or no physical barriers to dispersal.

Despite the presence of genetic differentiation between colonies and ocean basins in all pantropical booby species, we found evidence of gene flow across all but one physical barrier that we tested: the Indonesian Archipelago in red-footed boobies. Our dataset did not include samples from the immediate proximity of this land barrier, and so if gene flow has recently occurred across this barrier, it may not have been detectable in these genomes. Additional research with deep sequencing and more extensive sampling is required to fully address if gene flow has occurred between Indian Ocean and Pacific Ocean populations, or if this represents a ring species. This region holds promise for further genomic research into seabird speciation processes.

Given the reduced role of land barriers as isolating mechanisms demonstrated in our findings, the role of behavioral differences and genomic incompatibilities in maintaining species boundaries in boobies requires further investigation. Both blue-

footed boobies and brown boobies use carotenoid-mediated blue colored bare skin patches in mate choice (Torres and Velando 2003, Torres and Velando 2005, Velando et al. 2006, Montoya et al. 2018), but the role of bare-skin color patches in the other species in this clade, and how these color patches evolved, is unknown. However, divergent coloration in bare parts has been suggested to play a large role in the maintenance of seabird species boundaries (Pierotti 1987, Gay et al. 2009). Taken together, our findings indicate that further investigations into the role of sexual selection in generating and maintaining the diversity of species in this clade are crucial. Additional work is also needed to understand the role behavioral differences and genomic incompatibilities play in maintaining species boundaries, as well as the evolutionary consequences of introgressed regions of the genome in the three species pairs that demonstrated introgression. Speciation patterns across a variety of taxa highlight the importance of physical barriers in restricting gene flow. Land barriers clearly restrict some gene flow in seabirds, but the widespread patterns of gene flow demonstrated in our analyses indicate that other mechanisms facilitate speciation. Many dynamic processes shape the distributions of ocean-dwelling species, including ocean currents, nutrient levels, and global weather patterns (Faria et al. 2021). Our findings indicate that introgression may be especially prevalent in systems shaped by dynamic barriers to gene flow, and that biological factors, such as mating and behavioral preferences, may play a larger role.

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Figures

Figure 1: Sampling Locations and Species Identity of Each Individual Genome.

Sample locations are as follows: **blue-footed booby**: 1. La Plata, 2 & 3. Lobos de Tierra, 4. Isla San Ildefonso, 5. Seymour Norte, 6. Champion Island; **Peruvian booby**: 1. Isla Pajaros, 2 & 3. Lobos de Tierra, 4. North Chincha, 5. Lobos de Afuera, 6. Mazorca Island; **red-footed booby**: 1 & 2. Palmyra Atoll, 3. Genovesa, 4. Monito Island, 5. Aldabra Atoll, 6. Christmas Island; **brown booby**: 1. Palmyra Atoll, 2. Farallon de San Ignacio, 3. Monito Island, 5. Cape Verde; **masked booby**: 1. Herald Cay, 2. Johnston Atoll, 3. San Benedicto, 4. Monito Island, 5. Ascension Island, 6. Cosmoledo Atoll**;** **Nazca booby**: 1 & 2. Espanola, 3 & 4. La Plata, 5. Daphne, 6. Genovesa. Maps of full species distributions can be found in the supplement.

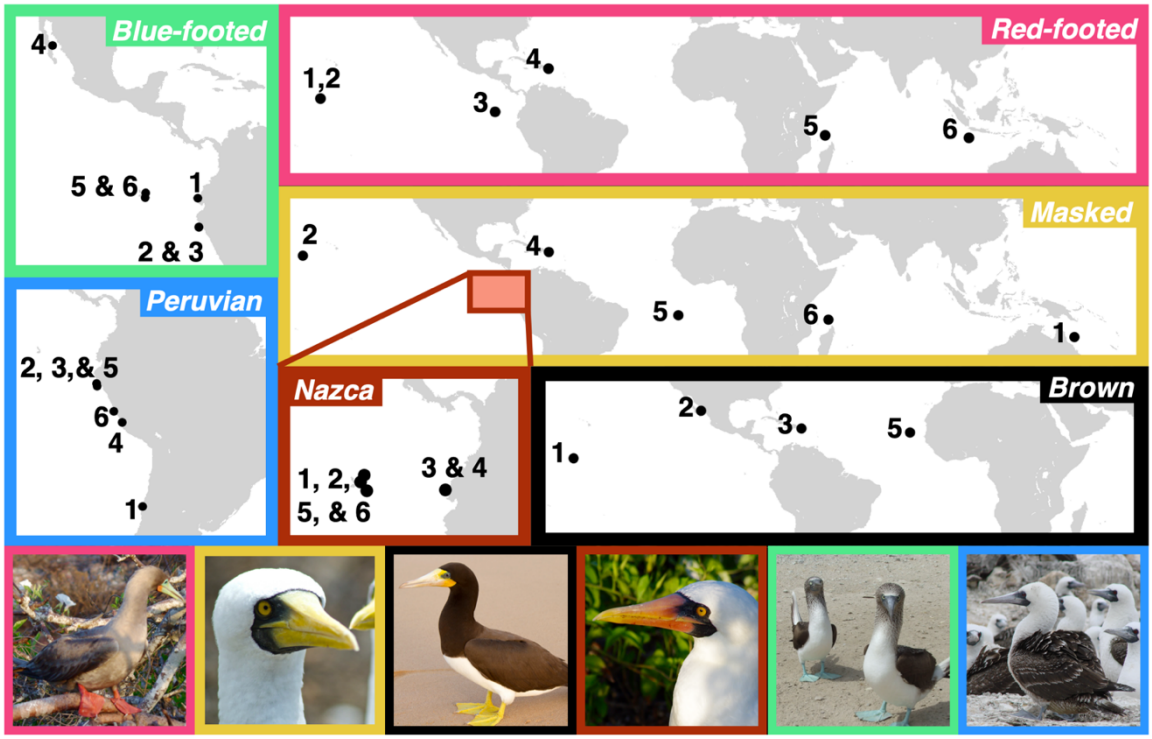


Figure 2: RAxML Best Tree (left) and Visualization of ABBA BABA Tests For Interspecific Introgression (right).

Best ML cladogram with nodes labeled with the bootstrap value. Tree was rooted with red-footed boobies as the outgroup, based on previous work (Friesen and Anderson 1997, Patterson et al. 2011). We display divergence dates on nodes representing species splits that were reproduced from relaxed clock estimates based on previously published multi-locus analyses (Patterson et al. 2011). Color bars indicate species designation. The ABBA BABA tests are visualized with arrows pointing from population 3 to populations 1 and 2, with solid arrows indicating which population demonstrated more shared genetic variation with population 3. Tests with no solid arrows were not significant (Table 1).

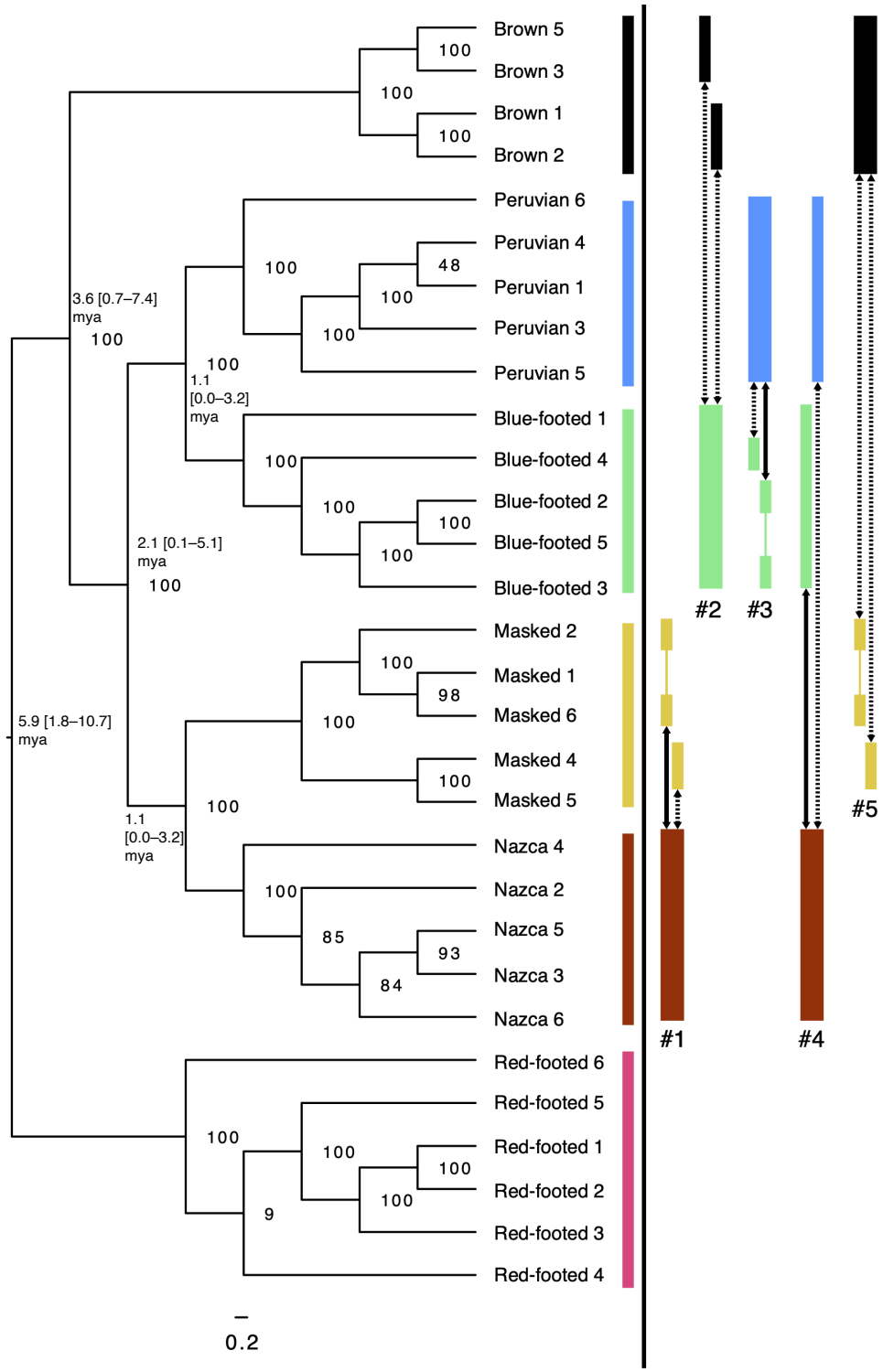
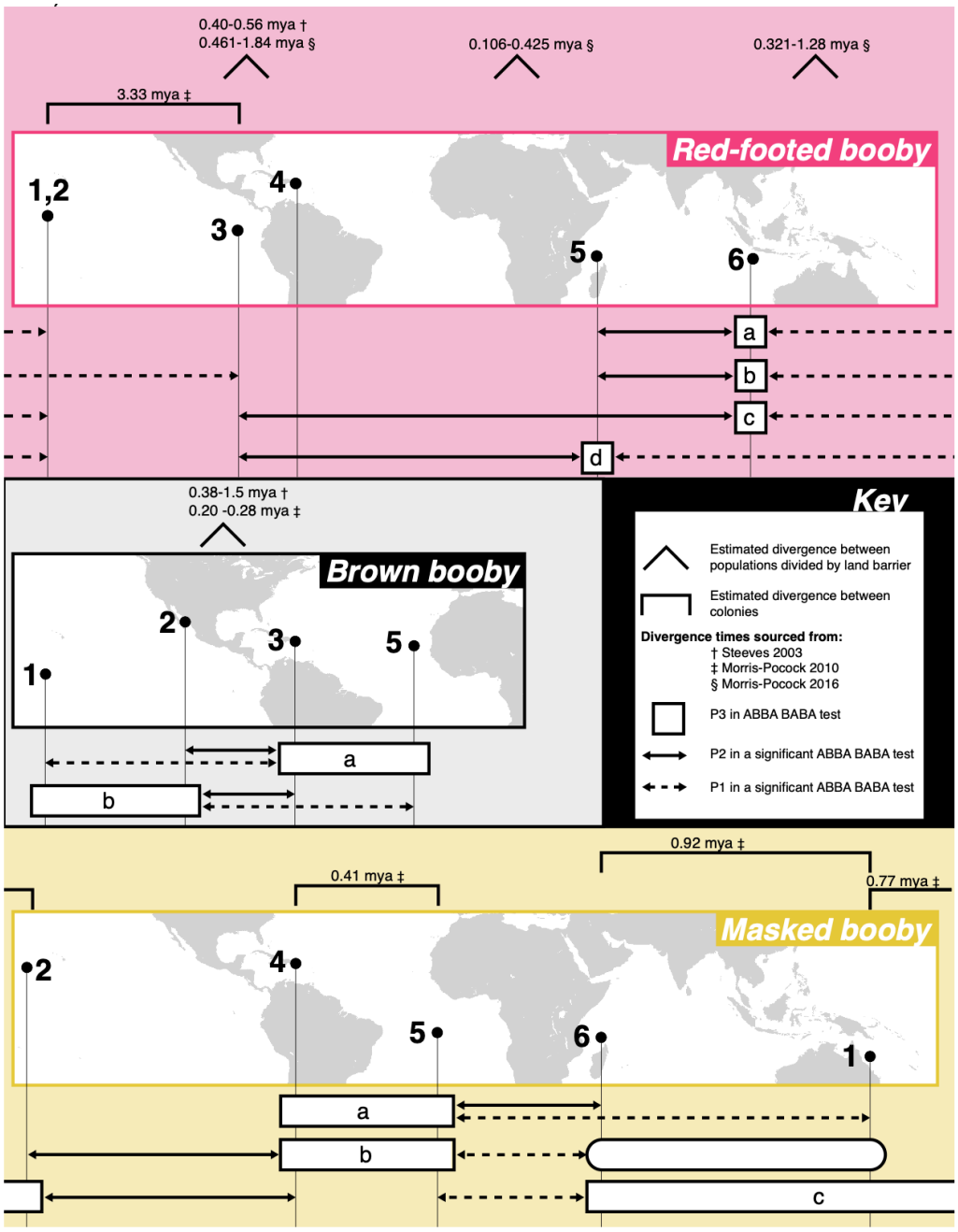

Brown 5
Brown 3
Brown 1
Brown 2
Peruvian 6
Peruvian 4
Peruvian 1
Peruvian 3
Peruvian 5
Blue-footed 1
Blue-footed 4
Blue-footed 2
Blue-footed 5
Blue-footed 3
Masked 2
Masked 1
Masked 6
Masked 4
Masked 5
Nazca 4
Nazca 2
Nazca 5
Nazca 3
Nazca 6
Red-footed 6
Red-footed 5
Red-footed 1
Red-footed 2
Red-footed 3
Red-footed 4
3.6 [0.7–7.4] mya
1.1 [0.0–3.2] mya
2.1 [0.1–5.1] mya
1.1 [0.0–3.2] mya
5.9 [1.8–10.7] mya
0.2
#1
#2
#3
#4
#5

Figure 3: Tests for Introgression (Intraspecies).

Visualized results of ABBA BABA tests for intraspecific introgression in pantropical boobies (red-footed boobies, brown boobies, and masked boobies). Population assignments for each test follow a typical D-statistic pattern of (((P1,P2),P3),O), where a boxed letter indicates P3, a solid arrow indicates P2, and a dashed arrow indicates P1; each test corresponds with the test of the same letter in Table S1. All tests were significant. Carets represent estimated times of divergence over land masses, and brackets represent dated times of divergence between colonies that are not separated by land masses, as determined in previous studies (†Steeves et al. 2003, ‡ Morris-Pocock et al. 2010, § Morris-Pocock et al. 2016).


0.40-0.56 mya †
0.461-1.84 mya §
0.106-0.425 mya §
0.321-1.28 mya §
3.33 mya ‡
Red-footed booby
1,2
4
3
5
6
a
b
c
d
0.38-1.5 mya †
0.20 -0.28 mya ‡
Brown booby
2
3
5
1
a
b
Key
Estimated divergence between populations divided by land barrier
Estimated divergence between colonies
Divergence times sourced from:
† Steeves 2003
‡ Morris-Pocock 2010
§ Morris-Pocock 2016
P3 in ABBA BABA test
P2 in a significant ABBA BABA test
P1 in a significant ABBA BABA test
0.92 mya ‡
0.41 mya ‡
0.77 mya ‡
Masked booby
2
4
5
6
1
a
b
c

Tables

Table 1: Hypotheses, Predictions, and Tests for Introgression (Interspecies)

Hypotheses, predictions, and test results of ABBA BABA tests for interspecific introgression among boobies (*Sula* spp.). Hypotheses and predictions are given above the tests as, for example, *H.1* and *P.1*. A Z score above 2.34 is considered significant here. Test 2a is significantly less than 0, which indicates that P1 and P3 share more genetic variation with each other than do P2 and P3. We ran Tests 2b–2f to further explore the unexpected results from Test 2a, which found that blue-footed boobies share more genetic variation with the allopatric Atlantic Ocean brown booby samples than with the sympatric Pacific Ocean brown booby samples. P1, P2, P3, and O list the sequences assigned to population 1, 2, 3 or the outgroup in an ABBA BABA test, in which P1 and P2 are sister, P3 is sister to both, and O is sister to all three. D, D sd, and D Z refer to the D test statistic, standard deviation, and Z score, respectively. Tests with significant values are bolded.

Test #	P1	P2	P3	O	D	D sd	D Z
H.1	*Introgression has occurred between Nazca and masked boobies.*						
P.1	*Nazca booby genomes share more genetic variation with Indo-Pacific masked booby genomes than they do with Atlantic/Caribbean masked booby genomes.*						
Test 1	**Masked (4,5)**	**Masked (1, 2)**	**Nazca (all)**	**Red-footed (all)**	**0.0686**	**0.0943**	**31.93**
H.2	*Introgression has occurred between brown and blue-footed boobies.*						
P.2	*Blue-footed booby genomes share more genetic variation with sympatric Pacific brown booby genomes than with allopatric Atlantic brown booby.*						
Test 2a	**Brown (3,5)**	**Brown (1,2)**	**Blue-footed (All)**	**Red-footed (all)**	**-0.3804**	**0.1251**	**-134.491**
Test 2b	Blue-footed (1,2,3,5)	Blue-footed (4)	Brown (1)	Red-footed (all)	0.035	0.8036	1.916

Test 2c	**Brown (3,5)**	**Brown (1,2)**	**Peruvian (All)**	**Red-footed (all)**	**-0.3583**	**0.2576**	**-61.644**
Test 2d	**Brown (3,5)**	**Brown (1,2)**	**Masked (All)**	**Red-footed (all)**	**-0.3671**	**0.2132**	**-76.137**
Test 2e	**Brown (3,5)**	**Brown (1,2)**	**Nazca (All)**	**Red-footed (all)**	**-0.3548**	**0.2646**	**-59.226**
Test 2f	**Peruvian (all)**	**Blue-footed (all)**	**Brown (1,2)**	**Red-footed (all)**	**-0.439**	**0.4605**	**-4.229**

H.3	*Introgression has occurred between Peruvian and blue-footed boobies.*						
P.3	*Peruvian booby genomes share more genetic variation with blue-footed booby genomes from regions of breeding overlap of the two species than they do with a blue-footed booby genome from the northern extent of the blue-footed booby breeding range.*						
Test 3	**Blue-footed (4)**	**Blue-footed (2,3)**	**Peruvian (all)**	**Red-footed (all)**	**0.1525**	**0.2007**	**33.181**

H.4	*Introgression has occurred between blue-footed and Nazca boobies.*						
P.4	*Nazca booby genomes and blue-footed booby genomes share more genetic variation than do Nazca booby genomes and Peruvian booby genomes.*						
Test 4a	**Peruvian (all)**	**Blue-footed (all)**	**Nazca (all)**	**Red-footed (all)**	**0.0152**	**0.2659**	**2.535**
Test 4b	**Blue-footed (4)**	**Blue-footed (5)**	**Nazca (all)**	**Red-footed (all)**	**0.0382**	**0.5023**	**3.338**

H.5	*Introgression has occurred between masked and brown boobies.*						
P.5	*Brown booby genomes from the Atlantic Ocean and Caribbean Sea share more genetic variation with sympatric masked booby genomes from the Atlantic Ocean and Caribbean Sea than they do with allopatric masked booby genomes from the Pacific Ocean.*						
Test 5	Masked (1, 2)	Masked (4,5)	Brown (3,5)	Red-footed (all)	0.0031	0.1642	0.831

CHAPTER 2

URBANIZATION DIFFERENTIALLY AFFECTS THE SPATIAL DISTRIBUTIONS OF TWO SYMPATRIC CONGENERS WITH SIMILAR ECOLOGICAL NICHES

Abstract

Aim

Urbanization has altered organisms and ecosystems around the world and will continue to do so into the foreseeable future. Although avian responses to urbanization at the community level have been well characterized, we lack species-level studies examining differences in spatial distributions in response to urbanization. We tested for differences in spatial distributions of two congeneric passerine bird species across an urban ecosystem, specifically in relation to a series of biotic, abiotic, and socioeconomic environmental variables.

Location

Arizona, United States of America

Taxon

Northern cardinals (*Cardinalis cardinalis*) and pyrrhuloxia (*Cardinalis sinuatus*), two similarly distributed and closely related songbird species.

Methods

We developed and deployed a new method for testing differences in spatial distributions between species using MaxENT, eBird, and structured bird-survey data across two

regions: the state of Arizona and across the Tucson metropolitan area. We then investigated potential urban-environmental factors that may similarly or differentially influence spatial distribution of (a) northern cardinal populations in two urban centers in the state (Tucson and Phoenix) and (b) both species in and around Tucson.

Results

We found that northern cardinals occur both further north in the state of Arizona and further into the city of Tucson than pyrrhuloxia. We also found that pyrrhuloxia are excluded from urban areas by habitat availability, but that they are positively associated with human development in the areas that they do reside. High-intensity urbanization has limited urban northern cardinal distributions, and their affinity for areas near open water may have facilitated their northward expansion across the state.

Main conclusions

Species distributions in response to habitat urbanization differ between even closely related species with very similar niches. Further research into the morphological, physiological, behavioral, and evolutionary differences between pyrrhuloxia and northern cardinals in Arizona may reveal the mechanisms that facilitate urban adaptation, expansion, or avoidance of some species but not of others.

Introduction

Human activities have rapidly changed natural landscapes throughout the globe over the last two centuries, and these changes continue to exert extreme pressures on free-ranging organisms (Allan et al. 2017, Elhacham et al. 2020, Gerten et al. 2019, Pörtner et al. 2023, Rosenberg et al. 2019, Seto et al. 2012, Watson et al. 2018). Cities contain novel resource distributions, temperature gradients, and ecological communities alongside unique anthropogenic disturbances that are driven by socioeconomic factors like wealth inequities (Chamberlain et al. 2019, Chen et al. 2021, Jenerette 2011, Kinzig et al. 2005, Schell et al. 2020, Sepp et al. 2017, Seress and Liker 2015). Not all species respond similarly to these changes, with some expanding (Clark 2017), contracting (Muñoz et al. 2021), or shifting their ranges (Arnold et al. 2021, Żmihorski et al. 2020) in response to human development.

Urbanization is broadly associated with declines in species richness (Afrifa et al. 2022, Chen et al. 2023, Haight et al. 2023**,** Hensley et al. 2019, Knapp et al. 2021, Lerman et al. 2021, Sol et al. 2020, Vasquez et al. 2022, Warren et al. 2019), but we lack an understanding of the more fine-scale, species-specific mechanisms underlying these patterns. The majority of comparative work into the effects of urbanization on species distributions is focused largely on comparisons of generalist versus specialist species (Abilhoa et al. 2017, Callaghan et al. 2019 and 2020, Devictor et al. 2007, Luck et al. 2010) or native versus introduced species (Humphrey et al. 2023, Lerman et al. 2020, Mills et al. 1989, Tsang et al. 2019). Comparative genetic, morphological, and behavioral studies have revealed that urbanization can have quite different effects on even closely related species, and those differences can illuminate some of the mechanisms underlying

species responses to urbanization (Fusco et al. 2021), e.g. differences in migration and dispersal rate (Markowski et al. 2021), body size (McNew et al. 2017), and feeding preferences (De León et al. 2018). To our knowledge, no study to date has compared spatial and habitat distributions between species with shared niches and evolutionary histories to test for differences in their responses to urbanization.

Species-distribution models can be constructed by using occurrence data of the species and spatial data of relevant environmental variables across the region of interest (Sillero et al. 2021). The probable spatial distribution of the species based on the association between occurrence and the environmental variables is called either a spatial distribution model (SDM) or an environmental niche model (ENM; see Peterson & Soberón 2012 for a terminology discussion). Contemporary methods for constructing these models include generalized additive models, maximum entropy models, random forest models, regularized regression models, and others, but MaxENT (a maximum entropy modeling approach, Phillips et al. 2004, 2006) is both widely used and among the top-performing spatial-distribution modeling approaches (Valavi et al. 2022). Although MaxENT has often been used to model species ranges under various human activities such as climate change (Nameer 2020), it has only rarely been applied to urban areas (but see Davis et al. 2012, Ito et al. 2020, Préau et al. 2018, Sallam et al. 2017, and Wiese et al. 2019), and never with a specific focus on differences in species distributions across urban areas. Similarly, MaxENT has not commonly been employed to compare species distributions (Espinosa et al. 2018) and has never been used within a hypothesis-testing framework to identify regions where species differ in their predicted occupancies. The software ENMTools implements tests to determine if two species have identical

distributions or if they have more similar distributions than would be expected by chance (Warren et al. 2010 and 2021), but no test exists to determine areas of significant difference between species.

Study system

Northern cardinals (*Cardinalis cardinalis*) and pyrrhuloxia (*Cardinalis sinuatus*) are two songbird species (Order Passeriformes: Family Cardinalidae) that are similarly distributed throughout much of the Sonoran Desert in the USA and Mexico and were estimated to have diverged around 6 million years ago (Provost et al. 2018, Barker et al. 2015, Hooper and Price 2017, Jetz et al. 2012, Kaiya Provost pers. comm). The southwestern northern cardinal subspecies (*C. c. igneus*) is a distinct population that is thought to have diverged from the nominate subspecies ~2.4 million years ago (Smith et al. 2011). However, there is not perfect geographic overlap between the two *Cardinalis* species. The range of *C. c. igneus* extends further north than that of *C. sinuatus*, and while both species are observed around the Tucson metropolitan area (pers. obs.), only northern cardinals are commonly seen around the Phoenix metropolitan area, although both are much more sparsely distributed than the eastern USA population of northern cardinals (Halkin et al. 2021). The northern expansion of the eastern U.S. population of northern cardinals, *C. c. cardinalis*, is believed to have only occurred after European colonization as a result of human-driven land-use changes (Halkin et al. 2021). The historic range of northern cardinals in the Sonoran Desert region was likely much more similar to that of pyrrhuloxia, with water and land use changes driving the northern expansion of this subspecies as well.

In this study, we combined community-science data (eBird; Fuller 2020, Sullivan et al. 2009) and structured survey data (Tucson Bird Count: Turner 2003; Central Arizona Phoenix Long Term Ecological Research Station Bird Survey, Warren et al. 2023) to compare the distributions of northern cardinals and pyrrhuloxia across two regions: the state of Arizona and across the Tucson metropolitan area. We also investigated the extent to which various biotic, bioclimatic, and socioeconomic environmental factors predicted distributions of both species in Tucson, and in northern cardinals between two cities in Arizona (Tucson and Phoenix). We outline the specific hypotheses and predictions tested in this study in Table 1. To our knowledge, this is the first comparative study of spatial distributions of congeneric species across an urban landscape, and our methods can provide a foundation for future investigations into the mechanisms underlying species responses to human disturbances.

Methods

All data will be made available on Dryad upon publication of this paper. The scripts used to run these analyses are available on GitHub at: https://github.com/dannyjackson/Spatial_Github

Species Occurrence Data

To determine presence of both bird species across Arizona, we used observational data from the Tucson Bird Count (TBC; 2001-2020, Turner 2003), the Central Arizona Phoenix Long Term Ecological Research Station Bird Survey (CAP LTER; 2000-2020, Warren et al. 2023) and eBird (2017-2021, Sullivan et al. 2009). We used all years

available from TBC, and we filtered eBird data to keep only 5 years of data because eBird has increased in popularity over time, and some of the datasets from earlier years may have been more biased by cultural differences in accessibility of eBird (Grade et al. 2022, Perkins 2020). We filtered all datasets to retain observations only during the breeding season, which we conservatively approximated in both species to be April and May (Halkin et al. 2021, Tweit and Thompson 2020, pers. obs.), and to keep only one observation per raster cell of each species (Johnston et al. 2021).

Environmental Data:

As predictors of species distributions, we used the following environmental variables: elevation (United States Geological Survey National Land Cover Database (USGS NLCD) Digital Elevation Model; USGS 2020), 19 bioclimatic variables (WorldClim database, Table S1, Fick and Hijmans 2017), tree cover (2016 USGS NLCD Tree Canopy Cover file, Homer et al. 2020; the 2019 USGS NLCD Tree Canopy Cover file is not yet available at the time of these analyses, as of May 10, 2023), percent developed imperviousness (2019 NLCD Percent Developed Imperviousness (CONUS) file, Dewitz and U.S. Geological Survey, 2021), land-cover variables (2019 USGS NLCD, Table S2, Dewitz and U.S. Geological Survey, 2021), and Median Household Income by census tract (U.S. Census Bureau 2020). These variables are commonly used in MaxENT models of avian distributions (i.e. Jenkins and Ha 2022).

We prepared and analyzed our data in R Statistical Software (v4.1.0; R Core Team 2021) using the packages dismo (Hijmans et al. 2011), raster (Higmans and Van

Etten 2012), rgdal (Bivand et al. 2015), rgeos (Bivand et al. 2017), ENMTools (Warren et al. 2010 and 2021), FNN (Beygelzimer et al. 2015), and leaflet (Graul and Graul 2016).

We split the NLCD file into separate tiff files, each representing 1 of the 20 variables in the NLCD file, excluding the 4 that exclusively pertain to Alaska, 1 that is irrelevant to the low desert (perennial ice/snow), and all 4 variables relating to urban development (Developed, Open Space; Developed, Low Intensity; Developed, Medium Intensity; and Developed, High Intensity). We excluded the urban-development variables because they are categorical representations of the percent of impervious surfaces in an area, which would be redundant with and less informative than the NLCD Percent Developed Impervious file. The cells in each of the files generated from the NLCD land-cover file represented either the presence of that variable with a 0 value, or the distance from that cell to the nearest cell containing that variable in meters. These represented distance to open water, barren land, deciduous forest, evergreen forest, mixed forest, shrub/scrub, grassland/herbaceous, pasture/hay, cultivated crops, woody wetlands, and emergent herbaceous wetlands.

We reduced the bioclimatic files to raster files representing their second and third principal components across the Arizona region because the original files were highly correlated with each other (Tables S3, S4, and S5). We excluded the first principal component because it was nearly perfectly correlated with the elevation file, and likely just represented variation in climate due to elevation. None of our final files had a final Pearson's correlation coefficient above 0.8 across the state of Arizona or the Tucson and Phoenix regions (Tables S6, S7, and S8).

Prior to analysis, each tiff file was reprojected to the World Geodetic System 84 (WGS 84) coordinate reference system, cropped to the boundaries of the state of Arizona, and resampled across the lowest resolution file, which were the bioclim variables with a 30-arc-second resolution (~1 km^2). We converted each file to an ASCII file, which is required for input into MaxENT. We then cropped these ASCII files for an analysis across the city of Tucson using the extent of a minimum longitude of -111.183682, a max of -110.720903, a minimum latitude of 32.034553, and a maximum latitude of 32.554540, and across the city of Phoenix using the extent of a minimum longitude of -112.584727, a max of -111.425540, a minimum latitude of 33.089419, and a maximum latitude of 33.885028. These were selected by determining the boundaries of the urban area from the US Census urban-area spatial file (U.S. Census Bureau 2020).

Analyses:

For the MaxENT analysis of each species across the entire state of Arizona, we randomly selected 10,000 points across the region for use as the background environmental conditions and randomly selected 50% of the observations for training data and used the remaining for model testing (Feng et al. 2017). Our analyses across Tucson and Phoenix used the same methods but only used 2,500 background points. We replicated these methods using subsets of observational data across the state using only eBird data, across Tucson using only eBird or only TBC data, and across Phoenix using only eBird or only CAP LTER data and we found similar results (data not shown). We only present the model that used the entire available data for observations. This produced our empirical models representing the distributions of northern cardinals and pyrrhuloxia

across the state of Arizona and across the city of Tucson, and the distribution of northern cardinals across the city of Phoenix.

We applied three tests of niche similarity, which test the null hypothesis that the two species distributions are randomly sampled from the same distribution of environmental variables and are effectively the same (Graham et al. 2004). We report these as *D*, *I*, and a rank correlation test, which test for significant differences in range of the species, and as *D env*, *I env*, and *rank correlation env*, which test for significant differences in the environmental niche of the species (Warren et al. 2008). These three test statistics have been shown to produce similar results, but we present the results of all three for consistency and comparability across the literature.

To test whether the two species differed in their distributions across the city of Tucson, we removed the species designations associated with each observation in the dataset of raw observations. Then, we randomly assigned each of the observations to one of the two species, keeping the number of total observations for each species equal to the true number of observations of that species. We then ran the MaxENT model using the same parameters as we did for our empirical analysis, and then subtracted the pseudoreplicate model for the spatial distribution of the probability of occurrence of pyrrhuloxia from the pseudoreplicate model for the spatial distribution of the probability of occurrence of northern cardinals. We repeated this process 1,000 times with different random permutations of the data to generate a null distribution of the differences between the distributions of the two species given the number of observations of each species. Finally, we subtracted the empirical model of the distribution of pyrrhuloxia from the empirical model of the distribution of northern cardinals and compared this file to the

1,000 null files. We used a significance level of 0.05, so we kept any cell in the empirical difference file that demonstrated an absolute difference between the probabilities of the two species that is greater than 950 of the null models. Every nonsignificant cell was converted to 0. We repeated this process using models across the state of Arizona to identify regions across the state where the two species differ. We visualized the spatial distribution of the significant differences in probability of occurrences of the two species in QGIS (QGIS 2023) and added major interstates to the map for visual reference (U.S. Census Bureau 2021).

We also compared permutation-importance values of the empirical MaxENT models. We used an arbitrary cutoff of a minimum of 5% to determine which variables contributed to the model, and then compared between species and regions to identify factors that differ in determining the distributions of the species.

Finally, to test for differences in the environmental variables associated with the distribution of each species, we extracted values from the raster files of each environmental variable for each observation of either species. We then ran t-tests on each environmental variable to compare for differences between species across the entire state of Arizona and across the Tucson region.

Results

Distributions of pyrrhuloxia and northern cardinals were significantly different across both Tucson and across the state of Arizona in all test statistics except for rank correlation of the Tucson model, which was nearly significant (p-value = 0.05; Table S9). Northern cardinals had a higher probability of occurrence in the city of Tucson than

pyrrhuloxia and had a higher probability of occurrence than pyrrhuloxia in the regions north of Tucson in the statewide analysis (Figure 1). However, the environmental niches were not significantly different between the two species in any of the test statistics, although several were nearly significant (Table S9).

Differences between species across Tucson

Five environmental variables explained a significant percentage of variation of both species' distributions across the city of Tucson (Table 2). These were the second and third principal components of the climate variables (northern cardinals: 19.8%, 18%; pyrrhuloxia: 20.1%, 7.3% respectively), elevation (northern cardinals: 5.1%, pyrrhuloxia: 8.7%), distance to evergreen forests (NLCD 42; northern cardinals: 20.9%, pyrrhuloxia: 16.1%), and distance to cultivated crops (NLCD 82; northern cardinals: 7.7%, pyrrhuloxia: 5.9%). None contributed only to the model of northern cardinals, and four contributed only to the model of pyrrhuloxia. They were surface imperviousness (8.3%), distance to barren land (NLCD 31, 7.2%), distance to deciduous forest (NLCD 41, 5.6%), and distance to grassland/herbaceous (NLCD 71, 5.2%).

Across Tucson, northern cardinals were more likely to be observed at areas of significantly greater impervious surface and greater canopy cover compared to pyrrhuloxia (Table 2). Northern cardinals were also closer to open water, barren land, deciduous forest, evergreen forest, and woody wetlands (NLCD 11, 31, 41, 42, 90), and further from shrub/scrub (NLCD 52) compared to pyrrhuloxia (Table 3).

However, in the response curves of the MaxENT model for Tucson (Figure S1), which depict the relationship of the species to each variable in the model, the distribution

of pyrrhuloxia was positively related to percent surface imperviousness, whereas the relationship between northern cardinal distribution and impervious surface area increased until 80% imperviousness, then steeply declined. Northern cardinals showed an affinity for proximity to open water, while pyrrhuloxia showed no relationship. Northern cardinals showed a negative relationship with barren land, and pyrrhuloxia showed a positive relationship with it. Northern cardinals showed an affinity for deciduous and evergreen forests, while pyrrhuloxia showed a negative relationship with the former but a positive relationship with the latter. Both species showed complex relationships with woody wetlands.

Differences between species across Arizona

In species-distribution models across the state of Arizona, five variables had significant permutation-importance scores for both species (Table 2): the second principal component of the climate variables (northern cardinals: 5.9%, pyrrhuloxia: 35.7%), elevation (northern cardinals: 27.6%, pyrrhuloxia: 21.8%), median household income (northern cardinals: 9.4%, pyrrhuloxia: 5.6%), NLCD 11 (distance to open water; northern cardinals: 10.3%, pyrrhuloxia: 7.3%), and NLCD 82 (distance to cultivated crops; northern cardinals: 22.4%, pyrrhuloxia: 18.9%). For the model of northern cardinals, NLCD 90 (distance to woody wetlands) was also significant (5.9%). No variables were significant only for the model of pyrrhuloxia.

Across the state of Arizona, northern cardinals were more likely to be observed at sites of significantly greater surface imperviousness and at lower elevation compared to pyrrhuloxia (Table 4). Northern cardinals were also closer to pasture/hay and woody

wetlands, but further from deciduous forest, evergreen forest, shrub/scrub, and cultivated crops, compared to pyrrhuloxia (Table 4).

In response curves output by the MaxENT model for Arizona, the two species followed very similar trends for all variables except for the third principal component of the climate variables, for which northern cardinals had a positive relationship and pyrrhuloxia had a complicated but negatively trending relationship (Figure S2).

For climate variables across Arizona (Table 4), compared to pyrrhuloxia, northern cardinals were found at higher Mean Diurnal Range (Clim 2), Temperature Seasonality (Clim 4), Max Temperature of Warmest Month (Clim 5), Temperature Annual Range (Clim 7), Mean Temperature of Warmest Quarter (Clim 10), Precipitation of Driest Quarter (Clim 17), and Precipitation of Coldest Quarter (Clim 19) (Clim 2, 4, 5, 7, 10, 17, 19). Northern cardinals were found at lower Isothermality (Clim 3), Mean Temperature of Wettest Quarter (Clim 8), Precipitation of Wettest Month (Clim 13), Precipitation Seasonality (Clim 15), and Precipitation of Warmest Quarter (Clim 18).

Differences between northern cardinals in Tucson versus Phoenix

In comparisons of the models of northern cardinal distributions between the cities of Tucson and Phoenix (Table 2, Figures 1, S3), two environmental variables – distance to evergreen forests (20.9% and 11.6% respectively) and to cultivated crops (7.7% and 10.2% respectively) – contributed significantly to the models across both cities. Four additional environmental variables had significant permutation importance values to the Phoenix model of northern cardinals but not to the Tucson model: surface imperviousness

(23.4%), distance from open water (NLCD 11, 15.8%), from deciduous forest (NLCD 41, 5.1%), and from grassland/herbaceous (NLCD 71, 11.2%).

In response curves output by the MaxENT model for each city, the distribution of northern cardinals differed in many variables between Tucson and Phoenix, but had similar response curves for percent surface imperviousness, and distances to barren land, deciduous forest, and evergreen forest (Figure S4).

Thirty of the thirty-two environmental variables associated with the distribution of northern cardinals differed significantly between the cities of Tucson and Phoenix (Table S10); the only ones that did not differ were surface imperviousness and distance from shrub/scrub (NLCD 52).

Discussion

We demonstrated that two closely related cardinal species do not have significantly different environmental niches, and that they differ in their probability of occurrence in both the highly urbanized center of Tucson and across the recently developed northern extent of Arizona. Northern cardinals are more prevalent than pyrrhuloxia in both regions. However, we also present evidence that pyrrhuloxia may be excluded from the urban center due to other environmental variables besides urbanization alone. Our findings further demonstrate that urbanization has similar effects on the same species in different cities (northern cardinals in both Tucson and Phoenix), but has different effects on even closely related species in the same city (pyrrhuloxia and northern cardinals in Tucson), and that fine-scale species differences underlie the ability of a species to persist in urban areas. Our work emphasizes the need for species-specific

studies to inform urban planning, specifically suggesting that urban residents and planners can reduce the negative impacts of human development on cardinal species by integrating a mixture of native canopy cover, shrub/scrub, and open ecosystems into the urban landscape. We also demonstrate that human land-use impacts extend beyond urbanization.

Are responses to urbanization similar between closely related species?

We found that effects of urbanization were similar within a species in different cities, but not between closely related species within the same city. Though neither species were present in the highest regions of surface imperviousness, northern cardinals were more likely to be observed in Tucson in areas with more impervious surface than were pyrrhuloxia. Interestingly pyrrhuloxia had a positive relationship with percent surface imperviousness in the MaxENT model response curves from Tucson. This suggests that pyrrhuloxia may be excluded from the city of Tucson by other environmental factors besides surface imperviousness, such as natural habitat availability (e.g. shrub/scrub and open habitat, Gould 1960), but that they may have an affinity for human development that occurs within their fundamental niche. Other studies of avian community structure have found differences in urban occupancies of closely related speces (e.g. Davis et al. 2012, Leveau et al. 2017), which suggests that species level differences may underlie urban community structures in many contexts. Our results emphasize that the effects of human development differ between even closely related species, with mid-to-high intensity urbanization having a greater impact on northern cardinals and low-intensity human development and resource management along the

suburban outskirts shaping the distribution of pyrrhuloxia, but with both species being largely impacted by habitat availability. Much of our understanding of avian responses to urbanization comes from only a few species (Fidino and Magel 2017), and our findings here demonstrate the need for species specific investigations to uncover the factors underlying species responses to urbanization. Further studies into the morphological, physiological, and genetic mechanisms that allow northern cardinals to persist in regions of higher urbanization than pyrrhuloxia will provide important clarity into the stressors affecting these species in Tucson.

Are responses to urbanization similar between the same species (northern cardinals) in different cities?

Despite differences in every other climate and land-use variable aside from canopy cover and distance from shrub/scrub, northern cardinals demonstrated a consistent response to urbanization in both Phoenix and Tucson. The consistency of the relationship between this species with canopy and shrub/scrub variables highlights the importance of these aspects of their environment to their persistence. These findings align with previous fieldwork that found northern cardinals require both open habitat and dense foliage within their nesting territories (Gould 1960). However, comparative studies of urban assemblages between cities in the southwestern U.S. found that overall avian communities differ between urban sites in cities (Hensley et al. 2019). Differences in percent impervious surface between cities may explain this seeming contradiction, since despite maintaining a similar association with urbanization between sites, the MaxENT model of northern cardinal distribution found more regions that excluded northern

cardinals in the urban center than the Tucson model found (Figures 1, S4). Our findings show that this species retains specific habitat needs across climate conditions that could be addressed with land use management plans along mid-to-high intensity urban areas that create interconnected patches of open space with dense foliage throughout the urban landscape. Several studies have found parallel responses in genetic variation to urbanization across multiple cities within the same species (Mueller et al. 2013, Mueller et al. 2020, Salmón et al. 2021, Winchell et al. 2023), and we show that spatial distributions are also consistently affected by urbanization.

What drives differences in species responses to urbanization?

We found differences between these species in their associations with land-cover variables in the city, and these differences affected the responses of these species to urbanization. Northern cardinals were found closer to open water and at higher levels of both impervious surface and canopy cover. The distribution of open water in the state of Arizona is heavily engineered, especially in urban areas, with irrigation systems, dams, and man-made lakes creating a novel pattern of water availability for the native wildlife (Colby and Jacobs 2007), and city planning in the desert creates novel matrixes of canopy coverage, with some sparse regions and some very dense regions (Nelson et al. 2021). This suggests that underlying differences in preferences for canopy density may allow northern cardinals to persist in the city, but not pyrrhuloxia, who demonstrate a greater affinity for shrub/scrub than northern cardinals (Gould 1960). Our work here expands upon previous findings in Phoenix that show effects of landscaping on avian communities (Warren et al. 2019), and we demonstrate the role of species differences in shaping urban

wildlife communities. Urban planning and landscaping that better integrate native shrub/scrub ecologies into the city may help reduce the exclusionary effects of urbanization on pyrrhuloxia. Our findings echo those from other systems that have found effects of various urban planning strategies on avian community structure (Benitez et al. 2021), but we emphasize the importance of understanding species specific associations with ecological variables within each urban area.

Our findings also illuminate climatic differences in ecological niches that might allow for differences in statewide distributions of these two species. Twelve climate variables differed between the species across the state of Arizona. Northern cardinals were found in areas with higher fluctuations in temperature at the daily, seasonal, and annual scales and with greater precipitation in the driest and coldest parts of the year. In contrast, pyrrhuloxia were found in areas with more precipitation in the wettest month and warmest quarter, which is the summer monsoon season. Pyrrhuloxia are also found in areas with higher mean temperatures during the wettest quarter, but northern cardinals were found in areas with higher mean temperature of the warmest quarter and maximum temperature of the warmest month. Although climate played an important role in shaping northern cardinal and pyrrhuloxia distributions at both citywide and statewide models, no climate variables differed significantly between observations of the two species across the city of Tucson. The overall combination of climatic factors may therefore be more deterministic for the occupancy of an area by either one of these species, and could be highly influenced by the climates of regions they historically occupied. For instance, evidence from several desert species show the effects of Pleistocene environmental processes on contemporary patterns of genetic variation (Provost et al. 2022), and our

methods in this study did not integrate historical climate data associated with these species. It is also possible that the two species depend on plants that are sensitive to different climatic phenologies, although no differential ecological specializations have yet been identified. Plant communities in Arizona are rapidly changing in response to climate change (Brusca et al. 2013) and if *Cardinalis* species show differences in associations with particular plants, this could explain differences in species responses both in urban areas and across the state.

Human impacts beyond urbanization

Only one land-use variable influenced the models of both species in all analyses: proximity to cultivated crops. This suggests that human land use beyond just city development plays a large role in shaping contemporary species distributions. Many studies of the effects of urbanization on avian wildlife compare populations along an urban to rural gradient, but our findings contribute to a growing body of evidence that suggest that rural land use changes may affect species alongside urbanization (e.g. Kumar and Kaur Kler 2021, Lazarina et al. 2020). Much of the research on cropland has focused on its effects on grassland species, which are often declining due to habitat loss (Pool et al. 2014, Scholtz et al. 2017). However, much like urbanization, croplands also facilitates range expansion of some species, with evidence of species differences within families (Veech et al. 2010), and further research into the complex effects of anthropogenic land use changes on avian community dynamics is needed at multiple scales beyond urbanization.

In the model of the entire Arizona region, three additional variables associated with human-influenced land use changes affected the distribution of both species: surface imperviousness, canopy cover, and proximity to open water. Proximity to open water also significantly contributed to the model of northern cardinal distribution across Phoenix, but not across Tucson, which further supports the notion that open water, and therefore the human engineering of it, is increasingly important for northern cardinals along the northern extent of their range in the state of Arizona. This aligns with evidence that riverways have historically been important for the ranges of northern cardinals (Smith et al. 2011). And the distribution of cultivated crops shapes much of the open space in areas along the outskirts of human development and within the habitats of these species. However, these variables also affected pyrrhuloxia distributions across Arizona, and observation locations of the two species only differed significantly from each other in their means of distance to cultivated crops, with pyrrhuloxia found closer to croplands than northern cardinals. This suggests that land-use change resulting from human activities underlies contemporary distributions of both species, leaving open questions about how and why northern cardinals have experienced a northward range expansion but not pyrrhuloxia. We did not differentiate between types of cropland, and crop diversity affects avian diversity in agricultural regions (Katuwal et al. 2022, Marcacci et al. 2021). Cropland management strategies could be differentially affecting these species. The difference in the response curves of the species to the climate variables, and only to climate variables, across the state of Arizona suggests that differences in the fundamental climate niches of the two species may have permitted northern cardinals but not pyrrhuloxia to expand their range in response to human development.

Summary

In conclusion, we demonstrate a novel method using permutations of species observations and MaxENT models to test for differences in the spatial distributions of species with similar niches. We found significant differences in urban occupancy between two congeneric species that share very similar ecological niches, and we also identify several anthropogenic and ecological variables associated with these distributional differences. The fact that environmental predictors of urban occupancy differ between northern cardinals and pyrrhuloxia in Arizona suggests that species differences in nesting and territory habitats have permitted northern cardinals to better adapt to areas of high human disturbance compared to pyrrhuloxia and highlights fertile ground for future comparative urban research. Our work emphasizes the importance of comparative studies in urban ecology as a method of understanding mechanisms underlying differences in species responses to human disturbances.

Acknowledgements

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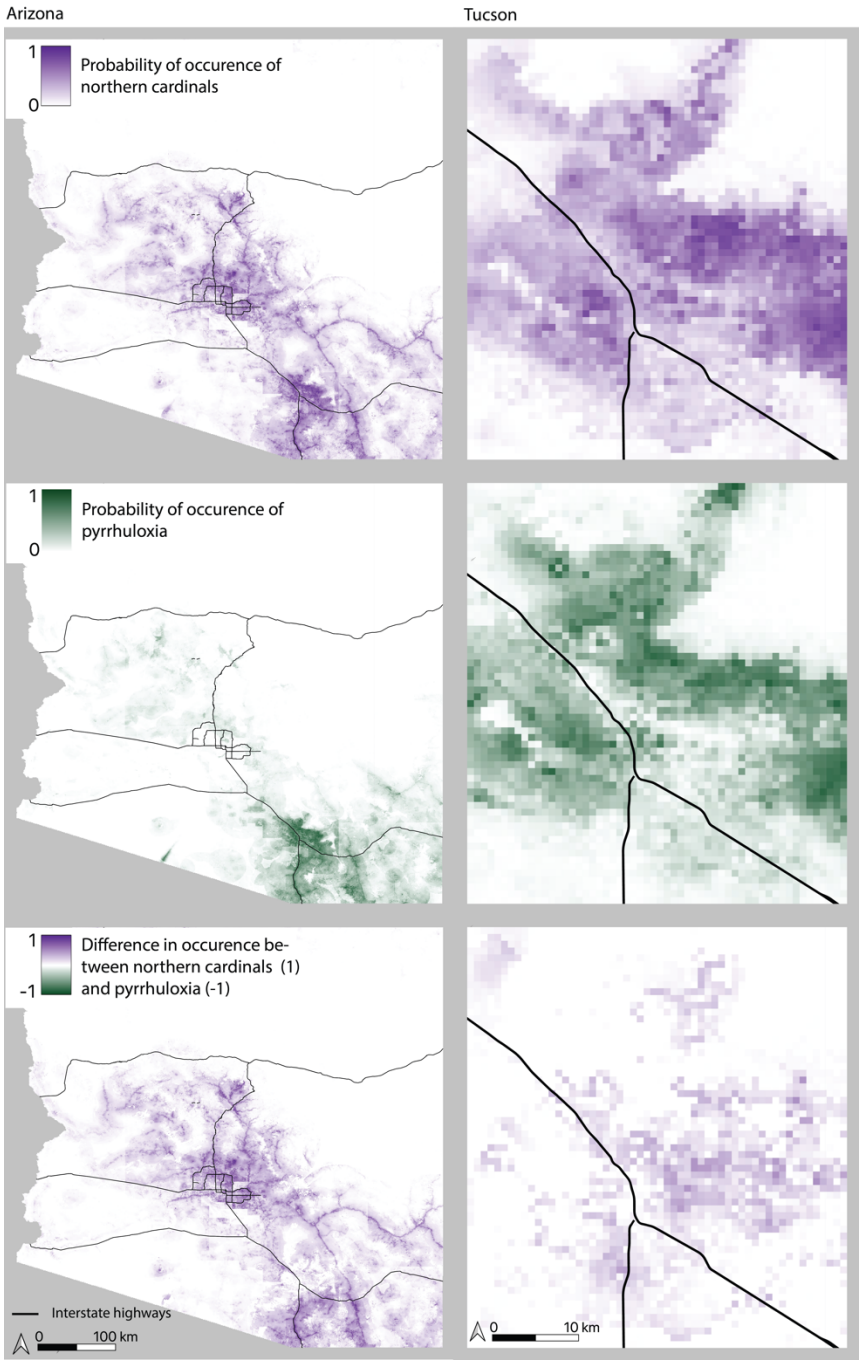
Figures

Figure 1: Predicted Probabilities of Occurrence of *Cardinalis* Species Across Tucson and Across Arizona

MaxENT models of the predicted probability of occurrence of northern cardinals in purple (top) and pyrrhuloxia in green (center), and the regions of significant difference between the two (bottom). Models across the Tucson region are on the top row and models across the Arizona region are on the bottom row. Interstates and major highways are displayed as a spatial reference. Each pixel represents a square kilometer.


Arizona
Tucson
1
0
Probability of occurence of northern cardinals
1
0
Probability of occurence of pyrrhuloxia
1
-1
Difference in occurence between northern cardinals (1) and pyrrhuloxia (-1)
Interstate highways
0
100 km
0
10 km

Tables

Table 1: Hypotheses and Predictions.

Test #	***Description***
H10.	The two species do not differ in their distributions across an urban environment.
H1A.	Given the pattern that generalist species with broader ecological niches tend to persist in cities better than their specialist counterparts (Warren et al. 2019), we predict that the species that has a broader statewide range (northern cardinals) will also have a broader range in the urban environment compared to their congener (pyrrhuloxia).
H20.	The same environmental variables will predict the distributions of the two species in both urban and rural habitats.
H2A.	Given general, observed/reported differences in the ranges and densities of these birds in urban versus rural environments in Tucson, the environmental variables that predict the distributions of the two species in an urban environment will be different from those across the broader range.
H30.	The environmental variables that predict the distributions of northern cardinals will not differ between cities (i.e. Phoenix v. Tucson).
H3A.	Given the differences in size, history, growth rates, and human population densities of Phoenix and Tucson, the environmental variables that predict the distributions of northern cardinals will differ between two cities.

Table 2: Permutation Importance of Environmental Variables in MaxENT Models

Variables representing the permutation-importance scores for northern cardinals and pyrrhuloxia from each empirical MaxENT model are shown. All units are percentages. Any variable with a permutation-importance score above 5% is considered to have significantly contributed to the model, and significant values are bolded.

	Tucson		**Arizona**		**Phoenix**
Variable	Northern Cardinal Permutation importance	Pyrrhuloxia Permutation importance	Northern Cardinal Permutation importance	Pyrrhuloxia Permutation importance	Northern Cardinal Permutation importance
ClimPC2	**19.8**	**20.1**	1.3	0.3	1.6
ClimPC3	**18**	**7.3**	**5.9**	**35.7**	2.6
Elev	**5.1**	**8.7**	3.8	0.7	0.5
imperviousness	4.3	**8.3**	**27.6**	**21.8**	**23.4**
MedianHouseholdIncome	2.7	1.2	4	1.6	4.2
canopy	0.6	0	**9.4**	**5.6**	1.1
NLCD 11: Open Water	3.2	4.6	**10.3**	**7.3**	**15.8**
NLCD 31: Barren Land	2.8	**7.2**	2.2	1.5	2.8
NLCD 41: Deciduous Forest	2.4	**5.6**	2.7	2.3	**5.1**
NLCD 42: Evergreen Forest	**20.9**	**16.1**	2.6	0.8	**11.6**
NLCD 52: Shrub/Scrub	2.4	2.5	0	0	4.3
NLCD 71: Grassland/Herbaceous	4.8	**5.2**	0.8	1.1	**11.2**
NLCD 81: Pasture/Hay	1.7	2.4	1.1	0.6	2.5
NLCD 82: Cultivated Crops	**7.7**	**5.9**	**22.4**	**18.9**	**10.2**
NLCD 90: Woody Wetlands	3.4	**5**	**5.9**	1.6	3.1

Table 3: Comparison of Environmental Variable Means for Northern Cardinal vs. Pyrrhuloxia Across Tucson

Means, standard deviations, and results of the t-test for the difference in means between the species are shown for each variable. Elevation is in meters. All units for NLDC files are in meters. Climate temperatures are in Celsius and precipitation values are in millimeters. A p-value of < 0.05 is considered significant, and rows with significant p-values are bolded.

Environmental variable	Northern cardinal mean	Northern cardinal SD	Pyrrhuloxia mean	Pyrrhuloxia SD	T	DF	P
Canopy	**0.69**	**3.47**	**0.16**	**1.01**	**3.55**	**727**	**< 0.01**
Elevation	826.54	84.23	831.11	81.04	-0.89	1004	0.37
Imperviousness	**22.77**	**25.81**	**18.72**	**23.40**	**-205.85**	**529**	**< 0.01**
Median Household Income	$79665.67	$21707.97	$80802.45	$21663.16	-0.85	987	0.40
NLCD 11: Open Water	**2585.76**	**1664.41**	**2875.35**	**1767.62**	**-2.71**	**954**	**0.01**
NLCD 31: Barren Land	**1197.14**	**997.49**	**1337.19**	**1232.12**	**-1.99**	**866**	**0.05**
NLCD 41: Deciduous Forest	**8782.49**	**7450.85**	**10951.92**	**8083.57**	**-4.48**	**942**	**< 0.01**
NLCD 42: Evergreen Forest	**6572.56**	**5870.97**	**7660.52**	**6903.74**	**-2.71**	**895**	**0.01**
NLCD 52: Shrub/Scrub	**87.33**	**204.22**	**44.96**	**118.38**	**4.24**	**991**	**< 0.01**
NLCD 71: Grassland/Herbaceous	2731.93	1590.36	2720.45	1634.67	0.12	972	0.91
NLCD 81: Pasture/Hay	12400.45	5715.35	13066.78	5646.68	-1.89	992	0.06
NLCD 82: Cultivated Crops	7445.95	3992.82	7650.06	3902.69	-0.84	997	0.40
NLCD 90: Woody Wetlands	**4358.03**	**2616.70**	**4673.02**	**2538.61**	**-1.97**	**1000**	**0.05**
Clim 1: Annual Mean Temperature	20.14	0.55	20.10	0.52	1.32	1017	0.19
Clim 2: Mean Diurnal Range	15.35	0.59	15.30	0.63	1.41	953	0.16
Clim 3: Isothermality	45.61	0.83	45.51	0.91	1.81	937	0.07
Clim 4: Temperature Seasonality	733.62	12.47	732.98	11.78	0.85	1011	0.40
Clim 5: Max Temp of Warmest Month	37.30	0.80	37.24	0.76	1.34	1008	0.18

Clim 6: Min Temp of Coldest Month	3.65	0.42	3.63	0.43	0.64	966	0.52
Clim 7: Temperature Annual Range	33.65	0.82	33.61	0.84	0.93	974	0.35
Clim 8: Mean Temp of Wettest Quarter	28.68	0.66	28.62	0.61	1.53	1022	0.13
Clim 9: Mean Temp of Driest Quarter	23.43	0.65	23.39	0.60	1.22	1020	0.22
Clim 10: Mean Temp of Warmest Quarter	29.22	0.68	29.15	0.61	1.72	1027	0.09
Clim 11: Mean Temp of Coldest Quarter	11.50	0.46	11.44	0.43	2.01	1011	0.05
Clim 12: Annual Precipitation	338.88	31.53	340.41	29.86	-0.80	1010	0.42
Clim 13: Precipitation of Wettest Month	60.74	4.71	60.95	4.40	-0.73	1016	0.47
Clim 14: Precipitation of Driest Month	5.59	0.64	5.59	0.61	-0.17	1008	0.87
Clim 15: Precipitation Seasonality	60.66	3.15	60.49	3.23	0.90	973	0.37
Clim 16: Precipitation of Wettest Quarter	154.39	12.75	154.68	12.05	-0.38	1011	0.71
Clim 17: Precipitation of Driest Quarter	21.60	2.21	21.61	2.09	-0.04	1011	0.97
Clim 18: Precipitation of Warmest Quarter	125.57	10.69	125.57	10.17	0.00	1009	1.00
Clim 19: Precipitation of Coldest Quarter	85.65	10.42	86.59	10.09	-1.48	1001	0.14

Table 4: Comparison of Environmental Variable Means for Northern Cardinal vs. Pyrrhuloxia Across Arizona

Means, standard deviations, and results of the t-test for the difference in means between the species are shown for each variable. Elevation is in meters. All units for NLDC files are in meters. Climate temperatures are in Celsius and precipitation values are in millimeters. A p-value of < 0.05 is considered significant, and rows with significant p-values are bolded.

Environmental variable	Northern cardinal mean	Northern cardinal SD	Pyrrhuloxia mean	Pyrrhuloxia SD	T	DF	P
Canopy	1.10	4.15	0.79	3.95	1.95	2129	0.05
Elevation	**934.16**	**294.09**	**1008.02**	**278.77**	**-6.69**	**2135**	**< 0.01**
Imperviousness	**14.55**	**22.91**	**11.66**	**20.56**	**3.48**	**2239**	**< 0.01**
Median Household Income	$68947.52	$27263.93	$69803.92	$23140.01	-0.90	2346	0.37
NLCD 11: Open Water	2943.13	2896.10	3071.06	2527.69	-1.24	2293	0.22
NLCD 31: Barren Land	3205.36	3753.89	2961.45	3584.97	1.72	2121	0.09
NLCD 41: Deciduous Forest	**18475.40**	**22291.06**	**10610.19**	**13780.84**	**11.94**	**2858**	**< 0.01**
NLCD 42: Evergreen Forest	**7527.39**	**9341.36**	**6468.55**	**7794.03**	**3.26**	**2379**	**0.01**
NLCD 52: Shrub/Scrub	**67.72**	**153.58**	**39.46**	**94.87**	**6.15**	**2859**	**< 0.01**
NLCD 71: Grassland/Herbaceous	1792.84	1642.24	1836.66	1859.83	-0.63	1830	0.53
NLCD 81: Pasture/Hay	**10850.86**	**8794.86**	**12422.67**	**8553.75**	**-4.69**	**2087**	**< 0.01**
NLCD 82: Cultivated Crops	**8392.41**	**7861.74**	**7942.76**	**6408.66**	**1.67**	**2424**	**0.01**
NLCD 90: Woody Wetlands	**3231.30**	**3261.02**	**4470.37**	**3716.73**	**-8.94**	**1820**	**< 0.01**
Clim 1: Annual Mean Temperature	18.91	1.99	18.82	1.77	1.37	2259	0.17
Clim 2: Mean Diurnal Range	**16.40**	**1.30**	**16.24**	**1.37**	**3.02**	**1950**	**< 0.01**
Clim 3: Isothermality	**46.83**	**2.65**	**47.36**	**2.46**	**-5.40**	**2177**	**< 0.01**
Clim 4: Temperature	**733.63**	**46.77**	**714.27**	**34.30**	**12.78**	**2619**	**< 0.01**

Seasonality							
Clim 5: Max Temp of Warmest Month	**37.04**	**2.21**	**36.34**	**1.86**	**9.05**	**2360**	**< 0.01**
Clim 6: Min Temp of Coldest Month	2.03	2.05	2.09	1.93	-0.79	2144	0.43
Clim 7: Temperature Annual Range	**35.01**	**1.87**	**34.25**	**1.51**	**11.92**	**2438**	**< 0.01**
Clim 8: Mean Temp of Wettest Quarter	**24.63**	**6.01**	**26.87**	**2.64**	**-14.05**	**2881**	**< 0.01**
Clim 9: Mean Temp of Driest Quarter	21.10	2.17	22.03	1.85	-0.40	2334	0.69
Clim 10: Mean Temp of Warmest Quarter	**28.03**	**2.27**	**27.66**	**2.07**	**4.46**	**2206**	**< 0.01**
Clim 11: Mean Temp of Coldest Quarter	10.28	1.85	10.36	1.56	-1.33	2353	0.19
Clim 12: Annual Precipitation	376.10	76.07	373.24	64.39	1.07	2352	0.29
Clim 13: Precipitation of Wettest Month	**69.06**	**23.27**	**76.20**	**21.63**	**-8.28**	**2172**	**< 0.01**
Clim 14: Precipitation of Driest Month	5.44	1.47	5.45	0.90	-0.15	2861	0.88
Clim 15: Precipitation Seasonality	**61.57**	**14.34**	**68.91**	**11.42**	**-15.13**	**2468**	**< 0.01**
Clim 16: Precipitation of Wettest Quarter	**169.27**	**49.80**	**185.04**	**44.74**	**-8.73**	**2236**	**< 0.01**
Clim 17: Precipitation of Driest Quarter	**25.57**	**8.43**	**23.43**	**4.21**	**9.17**	**2940**	**< 0.01**
Clim 18: Precipitation of Warmest Quarter	**137.06**	**46.55**	**153.33**	**40.70**	**-9.79**	**2289**	**< 0.01**
Clim 19: Precipitation of Coldest Quarter	**96.64**	**22.85**	**88.31**	**15.50**	**11.70**	**2744**	**< 0.01**

CHAPTER 3

DIFFERENTIAL EFFECTS OF URBANIZATION ON PLUMAGE AND MORPHOMETRIC TRAITS IN TWO CONGENERIC DESERT CARDINAL SPECIES

Abstract

Urbanization has already greatly affected wildlife and will continue to do so into the future, but we know very little about whether traits of closely related species respond similarly to this environmental change. This limits our understanding of how susceptible different species are to urban impacts. To address this gap, we tested the association between urbanization and morphological traits of two congeneric species, northern cardinals (*Cardinalis cardinalis*) and pyrrhuloxia (*C. sinuatus*), across the Sonoran Desert city of Tucson, Arizona over 137 years. We measured museum and field-collected specimens and applied a novel method to score urbanization over time. We found that urbanization reduced carotenoids in plumage traits in males, but in different traits in each species. For melanin-based plumage traits, urbanization was only associated with less saturated breast plumage in female pyrrhuloxia. Both urbanization and time of sampling were associated with shifts in feather and skeletal traits that could allow for improved flight maneuverability, and time was associated with wider bills in males of both species, which affects foraging, song, and heat tolerance. We demonstrate that urbanization has complex effects on traits involved in signaling, heat tolerance, foraging, and maneuverability. The effects of urbanization can differ even in closely related species that largely share a niche.

Introduction

Human activities have altered ecosystems globally, with only 23% of the terrestrial landscape (Watson et al. 2016) and 13% of the ocean still classified as wilderness (Jones et al. 2016), and with most wilderness areas under threat of global human impacts such as climate change, pollution, and habitat loss (Asamoah 2022). The impacts of urbanization on avian community structures have been extensively studied (Green 2003, Hostetler and Knowles-Yanex 2003, Litteral and Wu 2012, Hensley et al. 2019, Brown et al. 2022), but the effects of this ecological disruption on the phenotypic traits of bird species over time are less understood. Broad comparative studies at the community level can reveal overall trends in species abundance and distribution, such as the finding that bird species that persist in the city have a broader range of environmental tolerance than their rural congeners (Bonier et al. 2007, Palacio 2020, Tryjanowski et al. 2020). However, studies of traits from different populations or between closely related species can reveal the fine-scale, fitness-relevant mechanisms underlying these differences (e.g. Kern and Langerhans 2018, Winchell et al. 2018). This gap in our understanding highlights the urgent need for research on a diversity of morphological traits in response to urbanization.

Urban landscapes reshape selective pressures and environmental conditions for birds in a number of ways (Badyaev et al. 2008, Brown and Brown 2013, Hutton and McGraw 2016, Giraudeau et al 2014, McNew et al. 2017), and urbanization often removes correlations between color signals and behaviors or survival in birds (reviewed in Sepp et al. 2020). Urban stressors can be chemical pollution, noise, artificial light at night, human presence, novel patterns of resource distributions (reviewed in Isaksson

2018), and novel assemblages or abundances of species including pathogens (Giraudeau et al. 2014, Jiménez-Peñuela et al. 2019). Phenotypic differences across urban gradients can indicate which of these stressors have affected species, and therefore indicate which stressors might limit or shape urban patterns of species richness (Isaksson 2018). For instance, urban decreases in carotenoid-based plumage color can result from chemical pollution (Isaksson et al. 2005) or a reduction in diet quality (Isaksson and Andersson 2007), whereas increased in coloration in species inhabiting cities can result from invasive species that are rich in carotenoids (Jones et al. 2010, Baldassarre et al. 2022). Also, bill shape can be made longer and narrower due to reliance on anthropogenic food sources in bird feeders (Giraudeau et al. 2014) or shorter and wider due to increased competition and species interactions (Badyaev et al. 2008). And traits associated with maneuverability can be selected for as well, such as an increased need for quick, deft movements (i.e. longer tails and shorter wings) to navigate in a more complex and challenging vertical urban infrastructure (e.g. buildings, moving automobiles; Brown and Brown 2013). Although studies of phenotypic changes across an urban area can illuminate the particular stressors that can affect species, we do not know if the traits of sympatric congeners respond similarly to the same stressors in the same environment. Slight differences in congeneric species' responses to the same stressors could reveal intricate mechanisms of adaptation or acclimation associated with urbanization.

Population- and individual-level urban bird studies have largely focused on few species (e.g. house finches *Haemorhous mexicanus*, great tits *Parus major*; Heinen-Kay et al. 2021), and sample birds over relatively short time frames, often less than 30 years (Fidino and Magel 2017). This limits our understanding of the consistency of urban-

phenotypic trends across species, and of the responses of species over long periods of urbanization. To develop a more comprehensive framework for the impacts of human activities on species, new research methods that integrate species comparisons with sampling schemes across many decades are needed. Museum collections offer the ability to track phenotypes over time, as plumage and morphometric traits remain measurable across decades with proper methods (Burns et al. 2017). Despite this, preserved specimens have only rarely been used to analyze urban impacts on species over time, with only 12% of urban evolution studies accessing museum specimens (Shultz et al. 2021), and of those only one analyzed temporal changes in phenotype over time in both urban and rural populations of ten mammal species, rather than just in urban areas, and no such studies focused on bird species (Snell-Rood and Wick 2013).

Northern cardinals have recently emerged as a novel study system for understanding effects of urbanization on phenotype in birds. Studies of the nominate subspecies (*Cardinalis cardinalis cardinalis*), which is distributed throughout the eastern United States, have shown effects of urbanization on plumage coloration and body condition (Baldassarre et al. 2022) as well as song (Narango and Rodewald 2016). The northern cardinal is widespread throughout North America, with four genetic populations on the mainland of the continent that span urbanized areas (Smith et al. 2011). The second most predominant subspecies in the United States, *Cardinalis cardinalis igneus*, shares much of its range with its congener, the pyrrhuloxia (*Cardinalis sinuatus*; Smith et al. 2011); both are found throughout the Sonoran Desert and in the greater metropolis area of Tucson, Arizona.

The two species are easily distinguished by coloration (Figure 1), with male northern cardinals predominantly red with a black face mask and orange bill, male pyrrhuloxia predominantly gray with a red breast, face mask, crest, shoulder, and tail and orange bill, and females of both species similar to the coloration of the male pyrrhuloxia but with a buffy breast, black mask, and red-orange bill (female northern cardinal), or a gray breast and black mask (female pyrrhuloxia). The plumage of northern cardinals has been shown to have inter- and intra-sexual functions, although with variation between populations (Wolfenbarger 1999, Jawor et al. 2003, Jawor et al. 2004, Jawor and Brietwisch 2004, Jawor and Brietwisch 2006, Rodewald et al. 2011, Winters and Jawor 2017). Pyrrhuloxia also have a "parrotlike" curved bill, while northern cardinals have a more conical bill. Both species come to feeders in Tucson and generally occupy a similar niche with no observed differences aside from a slight preference for proximity to water in northern cardinals (Gould 1961), and little is known about their diet in the Sonoran Desert population (although see McAtee 1908). Northern cardinals are also present more in the urban center of Tucson than Pyrrhuloxia (Chapter 2). These slight differences in phenotype, but similarities in ecology/distribution allow for a unique comparative study on urban trait expression. Phenotypic differences between these species in plumage, bill morphology, wing length, and tail length in relation to urbanization could reveal which environmental stressors limit pyrrhuloxia's urban distribution more than that of northern cardinals and could highlight fine-scale differences in the mechanisms of urban adaptation between even closely related species.

We sought to determine if and how urbanization may affect the plumage and morphometric traits of northern cardinals and pyrrhuloxia. We present findings from

morphological traits of northern cardinals and pyrrhuloxia measured across 137 years between urban, urban outskirt, and rural areas across the state of Arizona. We model differences across urban areas to identify traits that have been impacted by human activities, as defined by proximity to urban areas. We expected to find that, if the effects of urbanization are consistent on similar species despite slight differences in ecological niche, the same traits would be impacted in similar ways by urbanization between these species. However, if urbanization differentially impacts closely related species, either the same traits would be affected but in different ways, or different traits altogether would be impacted by urbanization.

Methods

Data collection and processing

For the field portion of this study, we captured and sampled 13 northern cardinals and 12 pyrrhuloxia at Tucson residences from March to May of 2021 and 2022 using a mist net at feeders with black oil sunflower seed. We also took measurements from birds of both species from the University of Arizona's Bird Collection and from the University of Washington's Burke Museum of Natural History and Culture (Table S1), which together hold the majority of museum specimens collected in the state of Arizona and recorded in VertNet (Constable et al. 2010). We limited these to adult specimens collected in Arizona that were associated with reliable location data. We sampled 24 northern cardinals and 39 pyrrhuloxia from UAZ, and 11 northern cardinals and 5 pyrrhuloxia from UWBM, for a total of 48 northern cardinals (39 males and 9 females) and 56 pyrrhuloxia (34 males and 22 females) across both field and museum collections

(Figure S1). One male northern cardinal had outlier values for bill width and length and was the very first specimen measured (NOCA 001), so we excluded it from all morphology analyses but included it in coloration analyses.

Integumentary coloration was quantified using standard digital photography methods that have been validated in other carotenoid-colored passerine species (Giraudeau et al. 2012, Lendvai et al. 2013, McGraw et al. 2002). Our photographic methods did not capture variation in the ultraviolet (UV) portion of the spectrum, but UV and yellow-red reflectances are correlated in carotenoid-based plumages (Senar and Quesada 2006) and previous cardinal color studies have excluded UV quantification (Jawor and Breitwisch 2004). We took photographs with a Canon Rebel T3i and a Kodak color standard of two regions of each bird: their breast and their face in profile (Figure 1). We also photographed underwing plumage of the field specimens because this trait has been shown to vary with urbanization in female northern cardinals from New York (Baldassarre et al. 2022), but we could not evaluate underwing coloration of museum specimens due to the method of preservation. All photographs will be made available in our Dryad repository for other researchers to access upon publication of this paper.

Field photographs were taken in the shade under diffuse sunlight conditions to minimize shadow and included an 8” Tiffen Color Separation Guide with Grey Scale. Museum photographs were taken in front of a window with color standards to best replicate the natural sunlight conditions of the field samples within the constraints in place by the museums to preserve the specimen. From all field and museum specimens, we also measured length of the crest, wing, and tail to the nearest 1 mm with a wing rule; specifically, we measured the longest erect crest feather and the middle of the tail. We

also measured tarsus length as well as bill length and width at the nares to the nearest 0.1 mm with analog calipers.

We used Adobe Photoshop (24.0.1) to score plumage coloration from photographs. We analyzed the crest and face mask of each bird from photographs of the bird's head in profile, and the breast from a separate photo of that region of the bird. We obtained hue, saturation, and brightness values from each patch and from the red square of the Tiffen Color Separation Guide following methods from Giraudeu et al. (2012), except for the mask which is a gray-black shade and thus lacks a spectral peak from which to obtain hue. To standardize our measurements across light environments, we subtracted the value obtained from the Tiffen Color Separation Guide red standard from the value of the trait of interest. All methods were repeated on a second photo of each bird; intraclass correlation coefficients (ICC) were calculated to assess repeatability, and then values were averaged between the two photographs. ICC values ranged from 0.79 to 1.00, with a mean of 0.91 (Table S6).

Measuring the extent of urban impact

Dates for estimating degree of habitat urbanization for the capture locations of the cardinals ranged from 1885-2022, and the urban areas across Arizona changed drastically in size over those 137 years. To our knowledge, no studies have used long-term datasets to model urbanization of wild birds across over a century of samples, so we developed a novel protocol to measure urbanization here. We used a long-term dataset of urban extent, which contains raster files of hindcast modeled urban extents by decade (1880-1990), models from satellite nighttime light by year (1996-2009), or projected future

urban sprawl (2020) (Li et al. 2021). The model for 2020 projected urban areas based on past data, rather than on data from 2020, and it modeled urban extents under five different human development scenarios. Despite this, no difference was observed for our sampling locations between the different models. For each raster of urban extent, we generated a new raster file consisting of plots of the same resolution but where each cell represents the Euclidean distance (in m) of that cell to the nearest urban area in the original raster file using R Statistical Software (v4.2.2; R Core Team 2021). We then extracted this number from the cell at the same location as each bird-capture location within the raster file of the most recent year that predated the year of capture to generate a "Distance from Urban Area" score for each individual. If latitude and longitude data were not listed for certain museum specimens but there was a reliable description of capture location, i.e. "2.6 mi. E. of Arivaca, Pima Co., Arizona," then estimates of latitude and longitude were obtained using Google Maps. This attributed an urbanization score ranging from 0 meters (most urban) to 191,634 meters (most rural) to each specimen. Though this effectively captured variation in urbanization at the low end of the range (specimens at 0 meters were similarly all highly impacted by urbanization), cardinals caught at sites without any human development had wide ranging scores. To account for this, we binned the urbanization scores into three categories: Urban (Distance from Urban Area = 0 meters), Urban Outskirts (0 meters < Distance from Urban Area < 12,000 meters), and Rural (12,000 meters < Distance from Urban Area). We chose 12,000 as the cutoff because this was a natural break in our dataset (Figure S2), with no specimen having a distance to an urban area between 11,655 and 32,597 meters, and because all samples in this range appear to fit within the assigned categories when plotted

on a map (Figure S3). Analyses of females of both species lacked the sample size to retain these three urbanization categories, so for just the females of each species we lumped "Urban" with "Urban Outskirts" to create two categories: "Urban and Urban Outskirts" and "Rural." Human activity has increased over time in all areas, not just in urban areas, and museum specimen coloration might decay over time (Armetana et al. 2008). To account for this, we included the year of specimen collection in the model counting backwards with 2022 as year 0. For color traits, it is not possible to disentangle the effects of specimen decay from the effects of change over time on a population, but for morphological characteristics that are based on trait size (i.e. bill length), which should not decay, we inferred that the effects of year on that trait are a result of population change rather than specimen decay.

Data analyses

Dataframes were manipulated prior to analyses in Python using the packages pandas and numpy, and spatial files were processed in R with the packages raster (Hijmans), rgdal (Bivand et al. 2015), and terra (Hijmans et al. 2022). All analyses were run in R with the packages afex (Singmann et al. 2015), car (Fox et al. 2012), effects (Fox and Weisberg 2018, 2019), emmeans (Lenth et al. 2018), FNN (Beygelzimer et al. 2015), Hmisc (Harrell and Harrell 2019), HSAUR (Everitt et al. 2017), interactions (Long 2019), jtools (Long and Long 2017), lme4 (Bates et al. 2015), lmerTest (Kuznetsova et al. 2017), MASS (Venables and Ripley 2002), and tidyr (Wickham and Wickham 2017). Figures were plotted in R with ggbiplot (Vu, n.d.) and ggplot2 (Wickham et al. 2016).

We tested each morphological variable for normality using a Shapiro-Wilk test, and many were significantly not normal, so we then assessed correlations between all morphological variables by calculating the Spearman correlation coefficient for each pair of variables and the associated p-value. A high number of variables were significantly correlated with each other (Tables S2-S5). We chose to model each trait rather than the PCs of the traits despite these intercorrelations, as an understanding of the effect of urbanization on each trait is biologically meaningful and statistically appropriate since we modeled each trait separately.

For all color analyses, we ran linear models with the color trait as the dependent variable and urban category, tarsus length, year, and the interaction between year and urban category as fixed effects using the lm function from the R package "stats." We assessed multicollinearity using variance inflation factors (VIF) on each sex of each species using 3 as a cutoff, and as a result dropped both year and the interaction between urban category and year from analyses of northern cardinal females. No predictors exhibited collinearity for color analyses of northern cardinal males, pyrrhuloxia males, or pyrrhuloxia females. We assessed significance with an ANOVA on the fixed effects of the model using parametric bootstrapping with the Satterthwaite approximation for degrees of freedom applied to a restricted maximum likelihood (REML) fitted model (Luke 2017). We tested for differences in means following the ANOVA with a Tukey post-hoc test.

For all morphological analyses, we ran linear models with the morphological trait as the independent variable and urban category, tarsus length, year, and the interaction between year and urban category as fixed effects using the lm function from the R

package “stats.” Tarsus was included as a fixed effect in order to account for overall body size in the model. Significance was assessed using t-statistics output from the lm model. We again assessed multicollinearity using VIFs, and as a result dropped both year and the interaction between urban category and year from analyses of northern cardinal females. No predictors exhibited collinearity for color analyses of northern cardinal males, pyrrhuloxia males, or pyrrhuloxia females.

Results

Color traits

Northern Cardinal Males

Birds from different urbanization categories differed significantly in breast brightness ($F_2 = 4.056$, p = 0.027) (Figure 2, Table S7). Urban birds had darker breast plumage than rural birds (df = 31, p=0.024, CI = [11.59, 0.721]).

Year had a significant effect on crest brightness ($F_1 = 6.382$, p = 0.017) and face-mask brightness ($F_1 = 13.391$, p = 0.001), with older specimens having darker crests and brighter face masks (Table S7).

The interaction between year and urbanization category had a significant effect on face-mask saturation ($F_2 = 8.040$, p = 0.002), with more recent rural birds exhibiting a higher saturation than older rural birds, but more recent urban birds exhibiting a lower saturation than older urban birds, and no change over time for the urban outskirt birds (Figure S4, Table S7).

Northern Cardinal Females

We found no significant effect of urbanization category on the evaluated color traits for northern cardinal females (Table S8).

Pyrrhuloxia Males

Urbanization category had a significant effect on crest brightness ($F_2 = 5.220$, p = 0.013) (Figure 2, Table S9). Crests of male pyrrhuloxia were darker than those of rural birds (df = 24, p = 0.022, CI = [-14.461, -1.03]). Year had a significant effect on breast hue ($F_1 = 4.498$, p = 0.043) and breast brightness ($F_1 = 6.364$, p = 0.018), with older specimens having less red and brighter breasts.

Pyrrhuloxia Females

Urbanization category had a significant effect on breast saturation of female pyrrhuloxia ($F_1 = 4.448$, p = 0.050), with rural birds being more saturated than urban and urban-outskirt birds (df = 17, p=0.012, CI = [2.28, 16.2]) (Figure 2, Table S10). Year had a significant effect on face brightness ($F_1 = 4.802$, p = 0.043), breast hue ($F_1 = 15.643$, p = 0.001), and breast brightness ($F_1 = 10.674$, p = 0.005), with older specimens having brighter faces and brighter and less red breasts.

Size traits

For male northern cardinals, we found a significant effect of urbanization on tail length ($F_2 = 13.440$, p = <0.001) (Figure 3, Table S11). Urban birds have a longer tail than both urban-outskirt and rural birds (p < 0.001 and p = 0.016 respectively). Year had a significant effect on bill length ($F_1 = 5.71$, p = 0.023), bill width ($F_1 = 9.81$, p = 0.004),

and tail length (F_1 = 21.974, p = <0.001); over time, bills have gotten longer (β = -0.014) and wider (β = - 0.009), and tails have gotten longer (β = -0.139).

As with plumage, we found no effect of urbanization category on morphological traits of female northern cardinals (Table S12).

For male pyrrhuloxia, urbanization category had a significant effect on bill width (F_2 = 3.376, p = 0.049), with urban birds having a wider bill than urban-outskirt (p = 0.036, Figure 3, Table S13). Year had a significant effect on bill width (F_1 = 8.949, p = 0.006), and tail length (F_1 = 9.238, p = 0.005); over time, bills have gotten wider (β = -0.011) and tails have gotten longer (β = -0.068).

For female pyrrhuloxia, there were no effects of urbanization on morphological traits (Table S14), but year had a significant effect on wing length (F_1 = 5.481, p = 0.032), with older specimens having longer wings (β = 0.024) Figure 3, Table S14).

Discussion

We observed that for plumage and size traits, urbanization was not always associated with changes in the same traits between the species, but the effect of urbanization was in a consistent direction for all similar traits. Urbanization was consistently associated with reduced expression of carotenoid-based plumage coloration in males, although in different patches for each species. For feather traits relevant to flight, urbanization and time were both associated with increased maneuverability. And for bill morphology, time was both associated with larger bills.

In carotenoid-pigmented regions of both cardinal species, urbanization was associated with darker carotenoid-containing plumage. Darker coloration can be associated with greater carotenoid deposition in the bills of some birds, but no association between carotenoid content and plumage brightness has been documented (Butler et al. 2011), and further investigations into the mechanisms underlying this phenomenon are needed. The finding that male northern cardinal breasts are darker in the city of Tucson than in surrounding areas aligns with findings from Toledo, Ohio (Jones et al. 2010), but not with studies from Tampa, Florida (Leigh 2012) or Syracuse, New York (Baldassarre et al. 2022), neither of which found no association between breast brightness and urbanization, but the latter of which found an inconsistent association between urbanization and breast hue across years. We also found that carotenoid-pigmented crests in male pyrrhuloxia were darker in the city, indicating that urban environments have similar effects on different carotenoid-pigmented plumage regions in males of the two species. Breast coloration of both sexes of northern cardinals is used in mate choice (Jawor et al. 2003), and nothing is known about the signals involved in mate choice for pyrrhuloxia (Tweit and Thompson 2020), although given its conspicuousness and erection in a certain context, it would be surprising if the red crest of pyrrhuloxia served no social function.

We also found a negative association between urbanization and saturation of melanin-based buffy-gray breast plumage in female pyrrhuloxia. Melanin content should be correlated with hue, brightness, and saturation (McGraw et al. 2005), but none of the traits that we evaluated were significantly associated with urbanization for all three metrics. This finding therefore only weakly suggests an effect of urbanization on female

melanin and calls for further investigation. Nothing is known about the role of plumage color variation in pyrrhuloxia, but it has been suggested that melanin patches play more of a role in intrasexual communication (Badyaev and Hill 2000, Jawor et al. 2004), but evidence from horned larks (*Eremophila alpestris*) shows a role for melanin patches in mate choice (de Zwaan et al. 2019). Other avian melanin-containing color patches are affected by urbanization, such as the black ties of male great tits (*Parus major*; Yeh 2004, Senar et al. 2014). To our knowledge, ours is the first finding of a female-specific effect of urbanization on plumage color in birds. Given the lack of research on the role of melanin pigmentation in female avian social interactions and communication, especially in the context of human disturbance, this finding highlights a new avenue for future research.

Year of collection was associated with many color traits in our analyses, and much of this may be attributed to specimen degradation (Doucet and Hill 2009). In instances where temporal change was observed, older birds were less red and less saturated, but with some brighter features (male northern cardinal faces, male pyrrhuloxia breasts, female pyrrhuloxia faces and breasts) and some less bright features (male northern cardinal crests). Brightness values are negatively associated with carotenoid content of mallard beaks but are not associated with carotenoid content in house finch feathers (Butler et al. 2011). Urban birds also exhibited darker plumages in some carotenoid patches, so we demonstrate a general pattern of darker plumages over time and in urban areas for some patches in males of both species. Melanin-rich plumages can contain heavy metals (Isaksson et al. 2018), and heavy metal pollution is more prevalent in urban areas (Chatelain et al. 2021) and in association with mining (Rösner 1998),

which could explain why female pyrrhuloxia breast plumage (which is pigmented with melanin) is more saturated both in urban areas and in more recent specimens.

The congruous effects of time on bill morphology in northern cardinals and pyrrhuloxia suggest that these species experience the same pressures on this trait. In pyrrhuloxia, urbanization also had an positive relationship with bill size. This trend could result from selection related to foraging, as birds with larger beaks are faster at husking larger seeds (Nagy Koves Hrabar and Perrin 2002), or to heat tolerance, as larger bills allow for greater heat dissipation without a corresponding increase in evaporative water loss (Greenberg et al. 2012, Danner et al. 2017, Tattersall et al. 2017). The Sonoran Desert has become hotter and drier over the last century (Weiss and Overpeck 2005, Zhao et al. 2021), and urban areas are even hotter than the surrounding undeveloped areas (i.e. ‘heat island‘ effect; Brazel et al. 2007), so heat tolerance is a likely explanation. Urbanization is associated with larger bills in northern cardinals broadly across their range, with heat tolerance as a proposed explanatory factor (Miller et al. 2018). Studies of house finches in similar desert urban environments found contrasting patterns, as urban finches have longer but narrower bills (Badyaev et al. 2008, Giraudeau et al. 2014), with the selective effects of bird feeders listed as the probable factor. Both cardinal species frequent bird feeders around Tucson, often visiting the same feeders as house finches (pers. obs.). Other foraging factors could be affecting this trait, as both cardinal species consume many native seeds and, unlike house finches, commonly feed insects to their young (Halkin et al. 2021, Tweit and Thompson 2020), but we lack data on the foraging habits of these species in the Sonoran Desert, especially with respect to urbanization. It is also worth noting that an insectivorous species in New Zealand had shorter and wider

bills in association with long-term urbanization, although the mechanisms underlying this pattern are not known (Amiot et al. 2022). The effects of urbanization on insect prey species could also influence bill size, as urbanization favors small and medium beetles over larger beetles, although the trends across all insects are undocumented (Diamond et al. 2015), and reduced access to water from foraging on larger insects could interact with urban heat stress to select for wider bills.

The increased length of male northern cardinal bills, but not of male pyrrhuloxia bills, over time could result from a similar selective pressure for larger bills on both species but a contrasting pressure on bill length in pyrrhuloxia. Bill length in pyrrhuloxia could be limited by their unique, more decurved bill shape (Tweit and Thompson 2020), with selection favoring stouter bills to allow for a stronger bite (van der Meij et al. 2008), or it could be limited by foraging niche partitioning if their bill shape allows them to access resources that are unavailable to northern cardinals or other competitors. It could also be limited by physiological mechanisms, if the genetic mechanism for longer bills is constrained by an unexpected tradeoff. Regardless of the mechanisms underlying this difference, this finding demonstrates that foraging abilities could play a role in the success of these species in the urban environment.

Tail length increased in urban regions over time for male northern cardinals, and over time regardless of urban category for male pyrrhuloxia. Natural selection generally favors shorter tails in open environments, but longer tails in dense landscapes that require deft maneuverability (i.e. steering with a rudder; Thomas and Balmford 1995), such as urban environments. This may be species-specific, however, as great tits (*Parus major*) in the city have shorter tails than their rural conspecifics (Caizergues et al. 2021). An urban

environment may represent a more open habitat to forest-adapted species like great tits, but a relatively denser environment to these desert-adapted cardinals. Urban-rural comparisons of this trait in other northern cardinal populations that inhabit forested landscapes (i.e. across much of the eastern USA) will hopefully provide important context for the effects of human activity on this trait. The fact that tails of males of both species elongated over time and that wings of female pyrrhuloxia became shorter over time is also intriguing, as both traits are expected to allow for improved maneuverability (Swaddle and Lockwood 2003). There is no obvious trend of undeveloped areas of Arizona becoming more vegetatively dense in a relevant context for cardinals, though no areas are truly undisturbed and undeveloped in our study area. The increased heat and reduced water of the region due to recent climate change could be driving species to utilize more resource-rich and interannually stable habitats along riparian and human-developed areas even in more rural environments.

The sex-specific effects of morphological traits may result from differences in sample sizes that we were able to obtain between males and females, particularly for bill width and tail length. However, these patterns could also indicate sex-specific responses to urbanization, especially given the unique effect of urbanization on female pyrrhuloxia wing length. While both sexes of northern cardinals participate in territory defense (DeVries et al. 2020), males exert much more effort in territory establishment, intrasexual conflicts, and song performance (Gould 1961, Lemon 1968, Wilke 1995), and will feed females. However, females dominantly nest build, feed young, and develop the egg. It is unknown how these sex-specific behaviors interact with heat stress. The response of female pyrrhuloxia wing length to urbanization could suggest that selection for increased

maneuverability acts on wing length for female cardinals but tail length for male cardinals. We had a very small sample size of female northern cardinals, and it is possible that this prevented us from detecting a similar pattern for them as well.

Our findings demonstrate impacts of temporal and urban pressures on a pair of avian species and highlight the need for comprehensive studies that evaluate multiple social signals and morphological traits in the context of natural and urban ecologies. We show that two congeneric species with a similar ecological niche can experience different phenotypic changes in urban landscapes, but several traits also respond similarly between the species. We also identify a variety of traits that differ in the city that likely have social functions, and social selection on coloration may also be driving these differences in coloration. We also show that northern cardinal color signals are affected by urbanization in similar ways in a desert city as they are in the temperate deciduous forest cities of Ohio and New York, and we contribute novel findings of urban effects on bill length, bill width, and tail length that we hope will be examined in other cities and locations. Our findings fit into the broader landscape of exciting new urban ecological literature, and we look forward to new advances at the intersection of urban ecology, physiology, animal behavior, and evolutionary biology.

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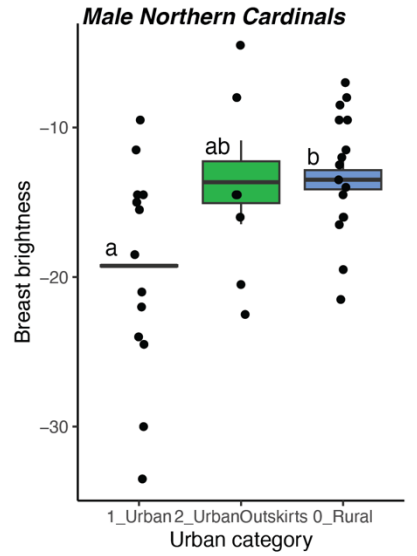
Figures

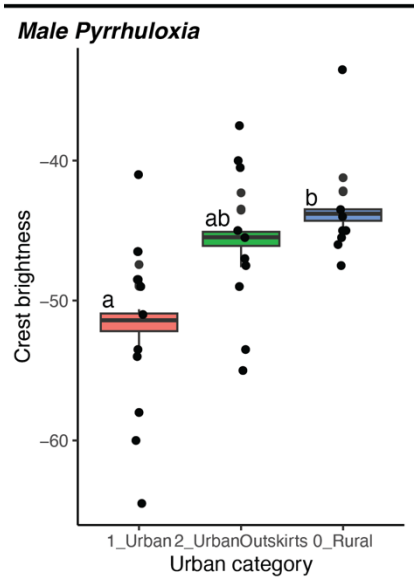
Figure 1. Head and Breast Photographs

Head and breast photographs of (from top to bottom) a male northern cardinal, a female northern cardinal, a male pyrrhuloxia, and a female pyrrhuloxia. Left photographs are from field caught birds, right photographs are from University of Arizona museum specimens.

Figure 2. Effects of Urbanization on Color Traits.

The predicted median and interquartile range based on the linear model of each color trait are presented as box plots, and actual trait measurements of the specimen are presented as points. We only present the four traits for which urbanization was significant and year was not, which were breast brightness for male northern cardinals, crest brightness for male pyrrhuloxia, and breast saturation for female pyrrhuloxia.


Male Northern Cardinals
Breast brightness
−10
−20
−30
a
ab
b
1_Urban
2_UrbanOutskirts
0_Rural
Urban category


Male Pyrrhuloxia
Crest brightness
−40
−50
−60
a
ab
b
1_Urban
2_UrbanOutskirts
0_Rural
Urban category

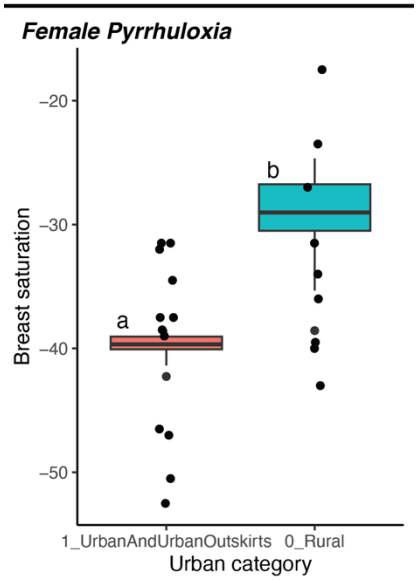
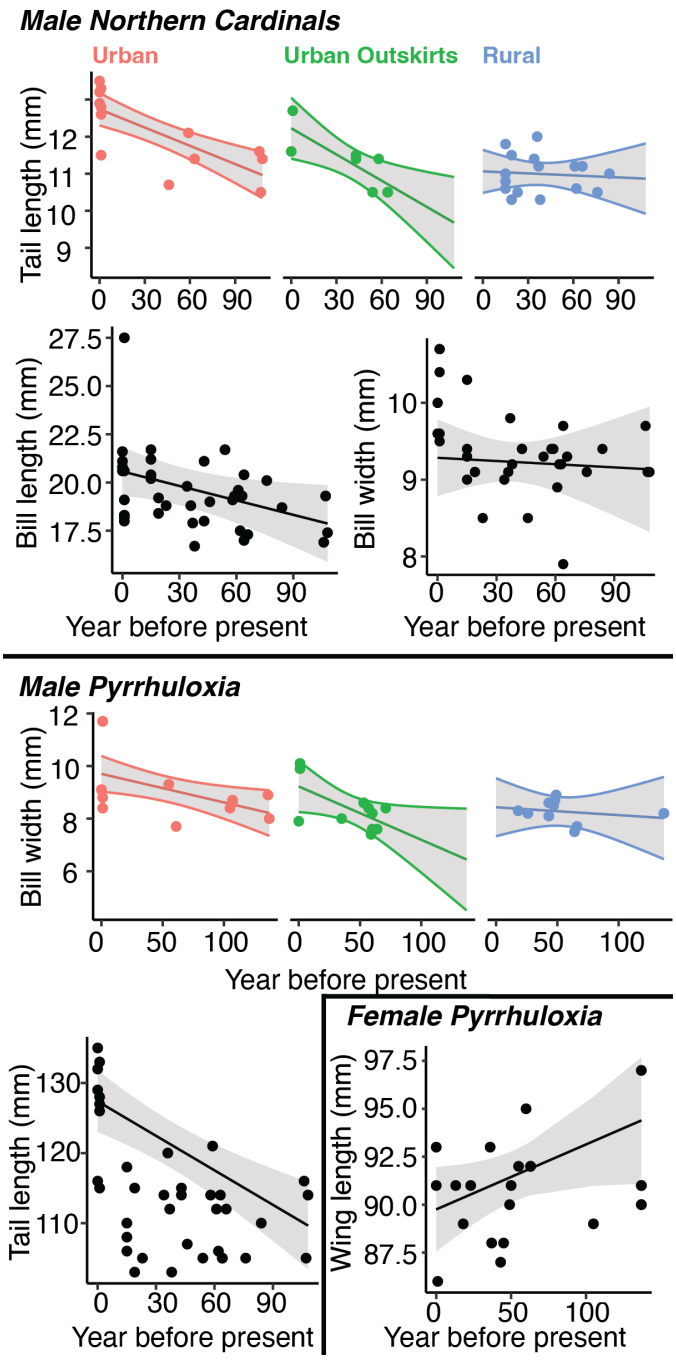

Female Pyrrhuloxia
Breast saturation
−20
−30
−40
−50
a
b
1_UrbanAndUrbanOutskirts
0_Rural
Urban category

Figure 3. Effects of Urbanization and Time on Trait Sizes.

The linear regressions with interquartile ranges of each trait size are presented with actual trait measurements of the specimen plotted as points. We only present the six traits for which both urbanization and year or the interaction between the two were significant, which were bill width, tail length, and bill length for male northern cardinals, bill width and tail length for male pyrrhuloxia, and wing length for female pyrrhuloxia.


Male Northern Cardinals
Urban
Urban Outskirts
Rural
Tail length (mm)
Bill length (mm)
Bill width (mm)
Year before present
Male Pyrrhuloxia
Bill width (mm)
Year before present
Female Pyrrhuloxia
Tail length (mm)
Wing length (mm)
Year before present

Tables

Table 1. Effects of Urbanization on Color Traits.

We only present results with either significant effects of urban category or of the interaction between urban category and year. Results of the full models can be found in the supplement (Tables S15-S18).

		Df	Sum Sq	Mean Sq	F value	Pr(>F)
Northern Cardinal Males						
Face						
	Saturation					
	Urban_categorical	2	106.90	53.44	0.44	0.65
	Year_Adj	1	74.80	74.81	0.62	0.44
	Urban_categorical:Year_Adj	2	1940.60	970.29	8.04	0.00
	Residuals	31	3741.10	120.68		
Breast						
	Brightness					
	Urban_categorical	2	291.56	145.78	4.06	0.03
	Year_Adj	1	0.00	0.00	0.00	1.00
	Urban_categorical:Year_Adj	2	17.73	8.86	0.25	0.78
	Residuals	31	1114.24	35.94		
Pyrrhuloxia males						
Crest						
	Brightness					
	Urban_categorical	2	348.87	174.43	5.22	0.01
	Year_Adj	1	93.07	93.07	2.78	0.11
	Urban_categorical:Year_Adj	2	2.36	1.18	0.04	0.97
	Residuals	24	802.05	33.42		
Pyrrhuloxia Females						
Face						

	Hue					
	Urban_categorical	1	9.53	9.53	0.51	0.49
	Year_Adj	1	116.96	116.96	6.21	0.02
	Urban_categorical:Year_Adj	1	90.28	90.28	4.79	0.04
	Residuals	17	320.23	18.84		
Breast						
	Saturation					
	Urban_categorical	1	283.95	283.95	5.44	0.03
	Year_Adj	1	232.01	232.01	4.45	0.05
	Urban_categorical:Year_Adj	1	60.48	60.48	1.16	0.30
	Residuals	17	886.79	52.16		

Table 2. Effects of Urbanization and Year on Trait Sizes.

We only present results with either significant effects of urban category, year, or of the interaction between the two. All results can be found in the supplement (Tables S19-S22).

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Northern Cardinal Males					
Bill Length					
Urban_categorical	2	0.95	0.47	0.25	0.78
Tarsus	1	4.18	4.18	2.19	0.15
Year_Adj	1	10.87	10.87	5.71	0.02
Urban_categorical:Year_Adj	2	0.72	0.36	0.19	0.83
Residuals	31	58.985	1.90		
Bill Width					
Urban_categorical	2	1.50	0.75	2.84	0.07
Tarsus	1	0.05	0.05	0.17	0.68
Year_Adj	1	2.58	2.58	9.81	<0.01
Urban_categorical:Year_Adj	2	0.88	0.44	1.67	0.20
Residuals	31	8.15	0.26		
Tail Length					
Urban_categorical	2	936.68	468.34	13.44	0.00
Tarsus	1	0.14	0.14	0.00	0.95
Year_Adj	1	765.76	765.76	21.97	0.00
Urban_categorical:Year_Adj	2	201.16	100.58	2.89	0.07
Residuals	30	1045.45	34.85		
Pyrrhuloxia Males					
Bill Width					
Urban_categorical	2	4.99	2.49	3.38	0.05
Tarsus	1	0.00	0.00	0.00	0.99
Year_Adj	1	6.61	6.61	8.95	0.01

Urban_categorical:Year_Adj	2	1.29	0.64	0.87	0.43
Residuals	27	19.95	0.74		
Tail Length					
Urban_categorical	2	94.99	47.50	1.46	0.25
Tarsus	1	12.66	12.66	0.39	0.54
Year_Adj	1	226.39	226.39	6.98	0.01
Urban_categorical:Year_Adj	2	124.82	62.41	1.92	0.17
Residuals	23	745.87	32.43		
Pyrrhuloxia Females					
Wing Length					
Urban_categorical	1	15.08	15.08	3.34	0.09
Tarsus	1	13.86	13.86	3.07	0.10
Year_Adj	1	24.75	24.75	5.48	0.03
Urban_categorical:Year_Adj	1	1.42	1.42	0.32	0.58
Residuals	17	76.76	4.52		

CHAPTER 4

URBAN-RELATED INTROGRESSION AND PARALLEL EVOLUTION IN TWO CLOSELY RELATED DESERT SONGBIRDS

Abstract

Urbanization has reshaped ecosystems globally and has created new selective regimes to which wild animals must adapt if they are to persist in the new urban ecosystem. Urban selection on genes involved in behavior, cognition, and immune function has been demonstrated in several avian species, but urbanization presents a variety of additional novel challenges (e.g. artificial light at night, noise pollution, novel resource distributions, etc.) that may differently shape gene flow and architecture. Studies of closely related, sympatric species may shed new light on how organisms experience genetic change in urban settings. Here we analyzed whole genome sequences of northern cardinals (*Cardinalis cardinalis*) and pyrrhuloxia (*C. sinuatus*) from urban and rural areas of Arizona, USA to test for the presence of urban-related parallel evolution and introgression. We identified 9 genes that appear to have undergone urban selection. 2 of the genes experienced parallel selection, and 4 show evidence of introgression from urban northern cardinals into urban pyrrhuloxia. Our findings emphasize the role of introgression in evolutionary responses to rapid environmental change, and identify light at night, energetic challenges, and pollution as driving stressors for urban adaptation.

Introduction

Urbanization can reshape natural selective environments and create novel challenges for wild species. Genetic variation underlying phenotypic changes associated with urbanization has been documented in several animal species, including genes that affect boldness behavior (van Dongen et al. 2015, Mueller et al. 2013), cognitive ability (Mueller et al. 2020), and immune function (Minias 2023, Pikus et al. 2021). Although the effects of urbanization can be consistent within a species across different urban environments (e.g., Mueller et al. 2013, Mueller et al. 2020, Salmón et al. 2021, Winchell et al. 2023), recent research has found differences in even the fine-scale mechanisms that underlie species responses to urbanization (Caizergues et al. 2022, McNew et al. 2017). Whether selection resulting from urbanization acts on the same genes in closely related species remains an unexplored area of research.

Urbanization can also disrupt species boundaries and facilitate gene flow (i.e., introgression) between species (Chafin et al. 2019, Grabenstein et al. 2022), either by increasing the rate of hybridization or by decreasing selection against hybrids (Grabenstein and Taylor 2018). If both species experience novel selection on the same traits in an urban environment, selection may act on one species while the other is unable to adapt, or both species might experience parallel selection on the same genomic regions (e.g. Winchell et al. 2023). If positive selection acts on an allele in only one species and hybridization occurs, selection has the potential to facilitate introgression (e.g., Jones et al. 2018).

Species that share a similar genetic background and a similar ecological niche, yet differ in their persistence in an urban environment, present a unique opportunity to

identify potential genetic mechanisms underlying species responses to urbanization. Northern cardinals (*Cardinalis cardinalis*) and pyrrhuloxia (*Cardinalis sinuatus*) both occupy the metropolitan area of Tucson, Arizona and largely share an ecological niche, but northern cardinals occupy more highly urbanized areas than pyrrhuloxia (Chapter 2). Both species demonstrate phenotypic responses to urbanization for traits involved in color signaling, heat tolerance, food handling, and flight maneuverability (Chapter 3). Although northern cardinals and pyrrhuloxia are 5.1 million years divergent (CI: 4.2 - 6.0 MY; Barker et al. 2015, Hooper and Price 2017, Kumar et al. 2017), they also infrequently hybridize. Two captive birds produced a viable hybrid offspring at the Sonoran Desert Museum (Griffiths 2022), and several sightings of hybrid individuals on eBird contain compelling photographic evidence, although no wild hybrids have ever been genetically confirmed, and genomic data from rural birds demonstrate no evidence of introgression (Kaiya Provost pers. comm.).

We compared whole genomes of northern cardinals and pyrrhuloxia to identify regions that are highly differentiated between urban and rural populations of each species and that may have undergone positive selection in the urban environment. We compared candidate regions between the species and identified regions that either underwent positive selection in both species or that likely introgressed between the species in the urban environment. To test between parallel selection and introgression, we conducted phylogenetic analyses of these candidate regions. Finally, we identified the functions of each candidate gene and compared functions within and between species to propose putative mechanisms underlying species responses to urbanization.

Methods

Tissue collection, DNA sequencing, and SNP filtering:

We collected blood samples from a total of 12 birds at residences around Tucson, Arizona spanning approximately 22 miles of the city: 6 northern cardinals and 6 pyrrhuloxia (Table 1, Figure S1). *Cardinalis* species in rural areas were difficult if not impossible to capture without destructive methods, so we also accessed muscle tissue samples from 12 birds (6 northern cardinals and 6 pyrrhuloxia) from the University of Washington Burke Museum and the Museum of Southwestern Biology. This produced a sample size of 6 per species per population (urban vs. rural), which is sufficient for identifying outlier regions between populations (Hahn 2019). We extracted DNA from each sample at the University of Colorado using a Qiagen DNeasy Blood & Tissue kit, and we measured DNA concentrations on a Thermofisher Qubit 3.0. Whole genome paired-end 150 base pair sequencing libraries were prepared and sequenced using the Illumina NovaSeq 6000 platform by Novogene, Sacramento, CA at approximately 4X coverage.

We trimmed raw sequence fasta files using Trimmomatic (Bolger et al. 2014) and analyzed for quality using FastQC (Andrews 2010). We aligned the trimmed sequence reads to the reference northern cardinal genome generated by the Birds 10,000 Genomes (B10K) Project genome (Feng et al. 2020; Assembly ASM1339721v1, GenBank ID GCA_013397215.1, 39,279 scaffolds, 72,526 contigs, N50 = 451.3 kb, L50= 559) using bwa mem (Li 2013). Then we sorted and indexed the resulting bam files using samtools (Li et al. 2009) and picard-tools ("Picard Toolkit" 2019), and called SNPs using BCFtools (Narasimhan et al. 2016). We filtered the resulting VCF using BCFtools to

filter out SNPs with a quality of 100 or lower, and VCFtools to filter out SNPs with a read depth of less than 2. This left 52,334 SNPs with an average read depth of 8.83 per sample (minimum of 6.73, maximum of 17.63). Finally, we used Plink (Purcell 2007) to filter out SNPs with a genotype quality score of less than 0.25, a minor allele frequency of less than 0.1, and to prune linked SNPs using a window size of 50kb, a step size of 5 SNPs, and an r^2 threshold of 0.5. This left 32,437 SNPs, which we used in all analyses.

Phylogenetic and Population Structure Analyses

We analyzed relationships between our samples using Randomized Axelerated Maximum Likelihood analyses (RAxML; Stamatakis 2014) and Principal Component Analyses (PCA). For RAxML, we converted the VCF to a Phylip file (Ortiz 2019) and ran RAxML under the GTRCAT model with 1000 random seeded bootstrap replicates using a Felsenstein ascertainment bias correction to account for the absence of non-variant sites. We visualized the tree in FigTree (Rambaut 2018) and we present the best tree with bootstrap support values. We conducted PCAs on the VCF in R using gdsfmt and SNPRelate (Zheng et al. 2012) on all samples and then separately on each species. We used the program STRUCTURE with 20,000 repetitions with clusters (K) of 2-6. We used VCFtools (Danecek et al. 2011) to calculate F_{ST} values between the two species, between urban and rural populations of each species, between urban populations of the two species, and between rural populations of the two species.

Tests for positive selection and introgression

To identify regions of the genome that have either recently undergone positive selection due to urbanization or that are associated with urban versus rural areas, we used six statistical approaches. First, we employed two approaches (F_{ST} scans and bayescan) that compare urban populations with rural populations of the same species. The other four approaches compute statistics within populations (i.e. on both urban and rural populations separately): nucleotide diversity $\theta\pi$ (Korunes and Samuk 2021, Nei and Li 1979), Tajima's D (Korneliussen et al. 2013, Tajima 1989), SweeD (Pavlidis et al. 2013), which detects recent selective sweeps based on allele frequencies, and OmegaPlus (Alachiotis et al. 2012), which detects recent selective sweeps based on linkage disequilibrium. We considered regions that were significant within urban but not within rural populations of the same species to be regions of relevance to selection within an urban environment. We explored 50 kb upstream and downstream of relevant regions to identify candidate genes, and we used snpEff to identify which of these genes have functional differences between individuals (Cingolani 2012).

We identified 9 genes (see below) within regions of interest that had functional mutations between urban and rural populations of one or both species. All of these were identified by either comparative analysis of urban and rural populations or by analysis of a single urban population. None of the analyses of rural populations resulted in the identification of any genes of interest with functional mutations. The remaining genes may have experienced positive selection on regulatory regions rather than on functional mutations. To test for the presence of parallel selection and introgression, we conservatively restricted our analyses to only the subset of genes that had evidence of functional mutations. We first filtered the VCF to contain only sites within each gene and

then analyzed each gene using PCA and, when possible, RAxML. RAxML requires more stringent filtering for missing data, and it could only be performed on 5 genes.

Results

Phylogenetic and Population Structure Analyses (RAxML, PCA, STRUCTURE)

In the RaxML analysis, each species formed a clade, but urban birds did not form a monophyletic clade in either species (Figure S2). In the PCA, the analysis that included all individuals first separated out species along PC1 (23.03% variation) but did not uncover any population structure along PC2 (4.05% variation; Figure S3). In the PCA of all northern cardinals, PC1 mostly separated urban samples from rural samples (9.91% variation) and PC2 spread rural samples but kept urban samples clustered together (9.45% variation). In the PCA of all pyrrhuloxia, PC1 separated urban birds from rural birds (23.03% variation) and PC2 mostly kept all urban and all rural birds clustered but spread one rural bird from the others. STRUCTURE recovered no clusters below the species level (Figures S3, S4).

We identified 162 fixed SNPs between the two species (genome-wide Weir and Cockerham weighted F_{ST} = 0.151), 9 fixed SNPs between urban and rural northern cardinals (Weir and Cockerham weighted F_{ST} of 0.006), and 0 fixed sites between urban and rural pyrrhuloxia (Weir and Cockerham weighted F_{ST} of 0.004). We identified 501 fixed sites between the rural samples of the two species (genome-wide Weir and Cockerham weighted F_{ST} = 0.152) and 347 fixed sites between the urban samples of the two species (genome-wide Weir and Cockerham weighted F_{ST} = 0.148).

Tests for Selection and Differentiation

We identified 34 genomic regions of interest, containing 31 total genes. Of these regions, F_{ST} scans identified 11, Tajima's D identified 15, nucleotide diversity identified 1, SweeD identified 3, and OmegaPlus identified 5. Two regions were identified by multiple programs: one by both nucleotide diversity and SweeD, and another by both SweeD and OmegaPlus. Only 3 regions were identified in both the urban northern cardinal population and the urban pyrrhuloxia population, containing 4 genes. snpEff found functional mutations in 3 of these genes (Table 2).

F_{ST} analyses between urban and rural populations of northern cardinals found an additional 3 regions of interest. Of these, snpEff only identified functional mutations in 1 gene (Table 2). F_{ST} analyses between urban and rural populations of pyrrhuloxia found an additional 8 regions of interest, which contained 8 genes. Of these, snpEff identified functional mutations in 3 genes (Table 2).

Tajima's D found 1 additional region of interest in urban northern cardinals which contained a single gene, and 9 additional regions of interest in rural northern cardinals which contained 9 genes. Of these, snpEff only identified functional mutations in the gene identified in the urban population (Table 2). Tajima's D also found 1 additional region of interest in urban pyrrhuloxia, which contained a single gene, and 4 additional regions of interest in rural pyrrhuloxia, which contained the genes 4 genes. Of these, snpEff again only identified functional mutations in the gene identified in the urban population (Table 2).

In total, we found 3 genes in both species with functional mutations that demonstrate evidence of either positive selection in an urban environment or

differentiation between urban and rural populations: CH037, HYDIN, and DLG2 (Table 2). We found 4 genes in just pyrrhuloxia: COL6A1, DCBLD2, RHO, and FXR1. And we found 2 genes in just northern cardinals: REXO1 and CTNNA3. Both HYDIN and CH037 function in the development of cilia and flagella, but while CH037 affects retinal development (Fahim et al. 2023), HYDIN affects lung function and sperm motility (Olbrich et al. 2012). Like CH037, RHO also affects the visual system (Nathans 1992). Both DLG2 and FXR1 influence brain functions (Branch et al 2022, Siomi et al. 1995), although FXR1 also influences muscle function (Mientjes et al. 2004). COL6A1 also affects muscle development (Pan 2003), and both CTNNA3 and DCBLD2 affect the heart muscle (Alhamoudi et al. 2021, Janssens et al. 2003). Finally, REXO1 affects RNA processing and can affect lung function (Herrera-Luis et al. 2022).

Of the 9 genes of interest, 4 displayed trends associated with introgression from urban northern cardinals into urban pyrrhuloxia: CH037, RHO, FXR1, and COL6A1 (Figure 2) and 5 did not (Figure S5). All 4 introgressed genes were identified in F_{ST} scans between urban and rural pyrrhuloxia (Figure 1). The RAxML analyses of RHO and COL6A1 found a clade containing all urban and rural northern cardinal samples as well as urban pyrrhuloxia samples, and a second clade containing just rural pyrrhuloxia samples (Figure 2). The PCA of all CH037 and RHO clustered urban samples together and separated rural samples of both species, and the PCA of COL6A1 clustered all northern cardinals together with a nearby cluster of urban pyrrhuloxia and a distant cluster of rural pyrrhuloxia (Figure 2).

Discussion

We demonstrate evidence of selection on 9 genes in two closely related songbird species. The functions of these genes include vision, lung function, muscle development, brain function, cancer, and RNA processing. Two of these genes demonstrate patterns consistent with parallel selection and affect sperm and lung efficacy and cognitive function. Four demonstrate patterns consistent with introgression from the urban population of northern cardinals into the urban population of pyrrhuloxia, two of which function in the visual system and two of which relate to muscle development. Our findings demonstrate that anthropogenic environmental change alters patterns of selection on congeneric species in similar ways and can even disrupt species boundaries and facilitate introgression.

Of the four genes that demonstrated evidence of introgression from northern cardinals into pyrrhuloxia, two are directly involved in the development of the visual system. Mutations in CH037 in humans are associated with cone-rod dystrophy and retinitis pigmentosa (Fahim et al. 2023), and RHO encodes the rhodopsin protein, which is the light-sensitive receptor protein in rod cells in the retina (Nathans 1992). CH037 was also highly differentiated between urban and rural northern cardinal populations, suggesting that this gene also experienced positive selection in the urban northern cardinal population. Several lines of evidence suggest that human activities affect the evolution of sensory systems in many fish species because eutrophication and turbidity can alter the light environment of aquatic environments. While urban environments present many novel visual challenges for species (Diamond et al. 2022), to the best of our knowledge we present the first evidence for selection on the visual system of a terrestrial

vertebrate as a result of urbanization (Kelley et al. 2018, but see Bloch 2015, which presents habitat-based selection on opsin genes in a non-urban context).

The other two genes that demonstrated evidence of introgression, FXR1 and COL6A1, both affect muscle development (Mientjes et al. 2004, Pan et al. 2003). Our previous work has shown that phenotypes in urban environments are associated with increased flight maneuverability compared to rural conspecifics (Chapter 3), highlighting the importance of flight function for urban survival. A similar selective pressure could be affecting muscle development in these species. FXR1 is also involved in brain development in humans because it is associated with fragile X syndrome (Siomi et al. 1995). Sex chromosomes in birds follow a ZW configuration, and it is unknown if this gene is also associated with effects in the avian brain.

HYDIN and DLG2 are the only two genes that experienced positive selection in urban populations of both species but do not show evidence of introgression. HYDIN plays a role in both sperm function and lung function, as it affects the development of cilia and flagella (Olbrich et al. 2012). REXO1 AND CTNNA3, which were identified as genes that underwent positive selection in northern cardinals but not pyrrhuloxia, are also involved in lung function (Herrera-Luis et al. 2022, Ong et al. 2013). Urban environments could create a novel selective pressure on lung function either due to higher levels of air pollution, which has affected lung physiology in other urban species (Isaksson et al. 2009, Torres-Blas et al. 2023). Or these genes could be responding to selection for increased energetics, the latter of which aligns with our previous findings on morphologies in the city. CTNNA3 is also associated with cardiomyopathy in humans (Janssens et al. 2003), as is DCBLD2 (Alhamoudi et al. 2021) and these genes could be

affected by selection for improved flight performance through their role in heart function. DLG2 is involved in brain function and also shows evidence of positive selection in urban burrowing owls (Mueller et al. 2020).

Our finding that urbanization is associated with selection on visual genes in these two species was surprising and intriguing. Because RHO specifically affects vision in dim-light environments, it suggests that nocturnal or crepuscular selection pressures (e.g. predator detection) or opportunities (e.g. foraging, singing, extra-pair mating; Kempenaers et al. 2010) may especially impact urban success of these species. Urbanization affects circadian rhythms of songbirds (Dominoni et a. 2013), and, at least in an Ohio population, urban northern cardinals fledge earlier in the day than their rural counterparts (Jones et al. 2023). Research in mammals suggests a shift toward nocturnalism among several carnivorous species in urban areas (Rtizel and Gallo 2020), which may impact nighttime selection pressures on the visual systems of prey species like songbirds. Alternatively, photoreceptors affect circadian rhythms (Prabhat et al. 2020, Senthilan et al. 2019), and this could instead represent selection allowing for the adaptation of the circadian rhythms of cardinals in the city. Some populations of great tits (*Parus major*) show selection on light-sensitive genes (Caizergues et al. 2022), but studies of several other passerine species in urban environments found no evidence of selection on visual genes (Mueller et al. 2013, Mueller et al. 2020, Salmón et al. 2021, Winchell et al. 2023). Why northern cardinals and pyrrhuloxia might experience unique selection on their visual systems in the urban environment requires further investigation. Future research into the urban behavior of passerines, and into the genotypic variation of

Cardinalis species across other cities, will provide important context for the effects of urbanization on native wildlife.

Figures

Figure 1: Weighted Weir and Cockerham F_{ST} Values of SNPs Comparing Urban and Rural Samples Within Species.

Plot A (top plot): F_{ST} Scan of Urban vs. Rural Northern Cardinals. Plot B (bottom plot): F_{ST} Scan of Urban vs. Rural Pyrrhuloxia. Genes within significant regions that snpEff identified as having functional mutations are annotated.

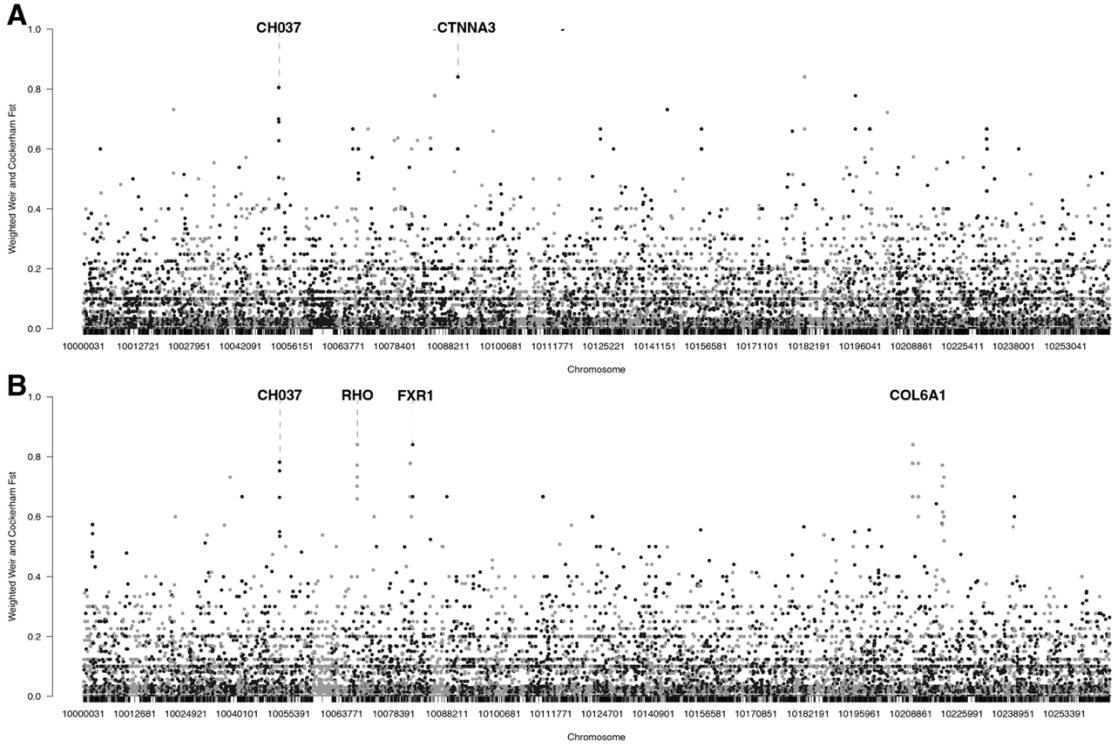
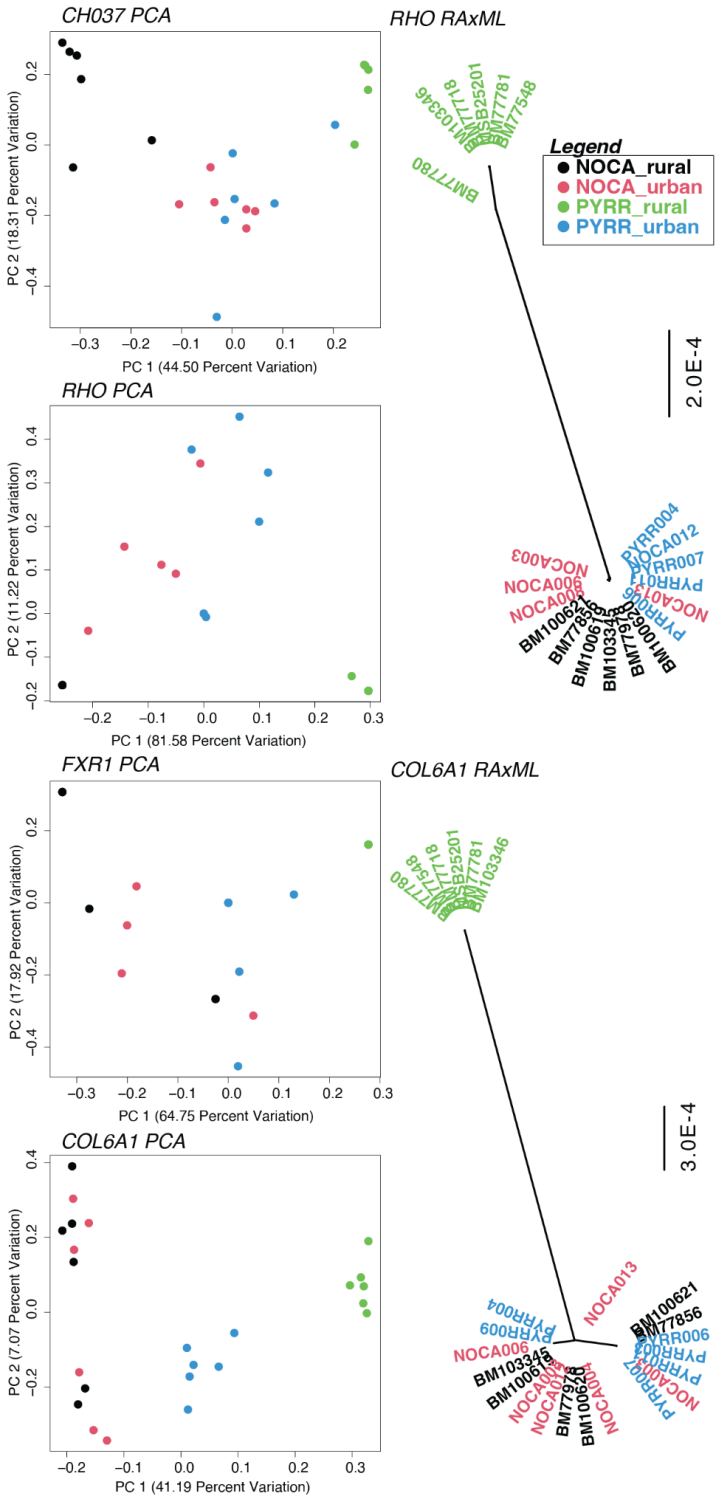


Figure 2: Phylogenetic Analysis of Introgressed Genes.

PCA plots (left) of CH037, RHO, FXR1, and COL6A1 show patterns of clustering between urban samples. Unrooted RAxML trees (right) of RHO and COL6A1 show that urban pyrrhuloxia samples are more closely related to all northern cardinal samples than to rural pyrrhuloxia samples at these genes.


CH037 PCA
PC 2 (18.31 Percent Variation)
PC 1 (44.50 Percent Variation)
RHO RAxML
Legend
NOCA_rural
NOCA_urban
PYRR_rural
PYRR_urban
2.0E-4
RHO PCA
PC 2 (11.22 Percent Variation)
PC 1 (81.58 Percent Variation)
FXR1 PCA
PC 2 (17.92 Percent Variation)
PC 1 (64.75 Percent Variation)
COL6A1 RAxML
3.0E-4
COL6A1 PCA
PC 2 (7.07 Percent Variation)
PC 1 (41.19 Percent Variation)

Tables

Table 1: Sample Information.

Sample	Species	Population	Source
NOCA_003	Northern cardinal	Urban	Field
NOCA_004	Northern cardinal	Urban	Field
NOCA_006	Northern cardinal	Urban	Field
NOCA_008	Northern cardinal	Urban	Field
NOCA_012	Northern cardinal	Urban	Field
NOCA_013	Northern cardinal	Urban	Field
PYRR_003	Pyrrhuloxia	Urban	Field
PYRR_004	Pyrrhuloxia	Urban	Field
PYRR_006	Pyrrhuloxia	Urban	Field
PYRR_007	Pyrrhuloxia	Urban	Field
PYRR_009	Pyrrhuloxia	Urban	Field
PYRR_012	Pyrrhuloxia	Urban	Field
UWBM_100619	Northern cardinal	Rural	University of Washington Burke Museum
UWBM_100620	Northern cardinal	Rural	University of Washington Burke Museum
UWBM_100621	Northern cardinal	Rural	University of Washington Burke Museum
UWBM_103345	Northern cardinal	Rural	University of Washington Burke Museum
UWBM_103346	Pyrrhuloxia	Rural	University of Washington Burke Museum
UWBM_77548	Pyrrhuloxia	Rural	University of Washington Burke Museum
UWBM_77718	Pyrrhuloxia	Rural	University of Washington Burke Museum
UWBM_77780	Pyrrhuloxia	Rural	University of Washington Burke Museum
UWBM_77781	Pyrrhuloxia	Rural	University of Washington Burke Museum
UWBM_77856	Northern cardinal	Rural	University of Washington Burke Museum
UWBM_77978	Northern cardinal	Rural	University of Washington Burke Museum
MSB_25201	Pyrrhuloxia	Rural	Museum of Southwestern Biology

Table 2: Genes of Interest

Gene name	Program(s) that identified the gene	Species	Function
HYDIN	OmegaPlus and SweeD	Northern cardinals and pyrrhuloxia	Axonemal central pair apparatus protein. Cilia and flagella development. Especially important for sperm function and lung function.
DLG2	Tajima's D	Northern cardinals and pyrrhuloxia	Discs large MAGUK scaffold protein 2. Cognitive function and neuronal development.
CH037 (synonym of CFAP418)	F_{ST}	Northern cardinals and pyrrhuloxia	Cilia and flagella associated protein 418. Vision, associated with retinal dystrophy. Can affect both cones and rods.
FXR1	F_{ST}	Pyrrhuloxia	FMR1 autosomal homolog 1. Brain function (fragile X syndrome), muscle development, and cancer
REXO1	Tajima's D	Northern cardinals	RNA exonuclease 1 homolog. RNA polymerase II transcription, cervical cancer cell proliferation and progression. Putatively involved in asthma.
RHO	F_{ST}	Pyrrhuloxia	Rhodopsin. Night vision
CTNNA3	F_{ST}	Northern cardinals	Catenin alpha 3. Cell-to-cell adhesions. Cardiac myopathy, growth, testicular development, asthma
COL6A1	F_{ST}	Pyrrhuloxia	Collagen type VI alpha 1 chain. Muscle development
DCBLD2	Tajima's D	Pyrrhuloxia	Discoidin, CUB and LCCL domain containing 2. Cancer (reduces metastatic properties of cancer cells)

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APPENDIX A

CHAPTER 1 SUPPLEMENTARY TABLES AND FIGURES

Figures

Figure S1. Principal component analysis of all samples: red-footed (RFBO), brown (BRBO), masked (MABO), Nazca (NABO), blue-footed (BFBO), and Peruvian (PEBO) boobies.

Principal component 1 separated red-footed boobies from all other species, and principal component 2 separated brown boobies from the 4-taxon clade of masked, Nazca, blue-footed, and Peruvian boobies (PC1 = 6.54 percent variation, PC2 = 5.63 percent variation).

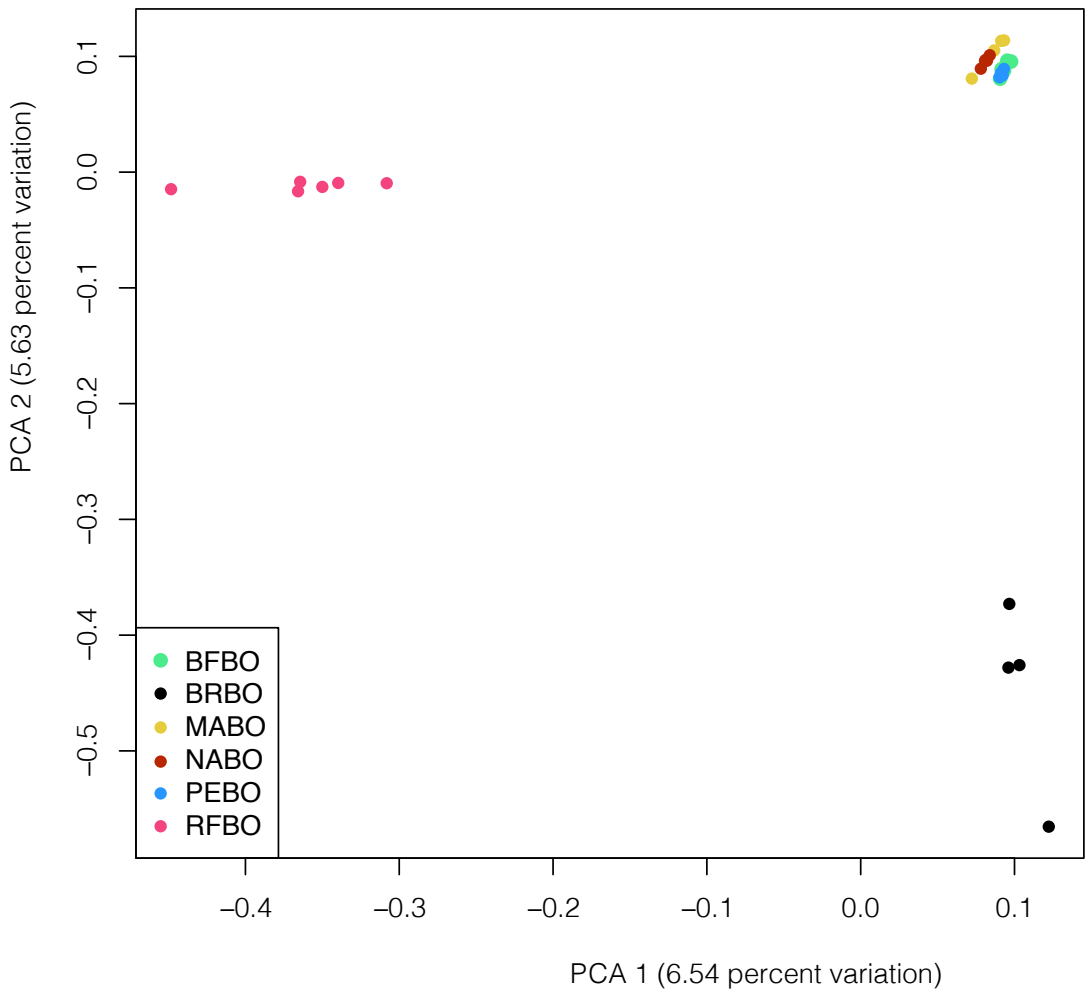

PCA 2 (5.63 percent variation)
0.1
0.0
−0.1
−0.2
−0.3
−0.4
−0.5
BFBO
BRBO
MABO
NABO
PEBO
RFBO
−0.4
−0.3
−0.2
−0.1
0.0
0.1
PCA 1 (6.54 percent variation)

Figure S2. Principal component analysis of the four-taxon clade: masked (MABO), Nazca (NABO), blue-footed (BFBO), and Peruvian (PEBO) boobies.

Principal component 1 separated the two pairs of sister species from each other, and principal component 2 separated masked boobies from Nazca boobies (PC1 = 7.94 percent variation, PC2 = 6.51 percent variation).

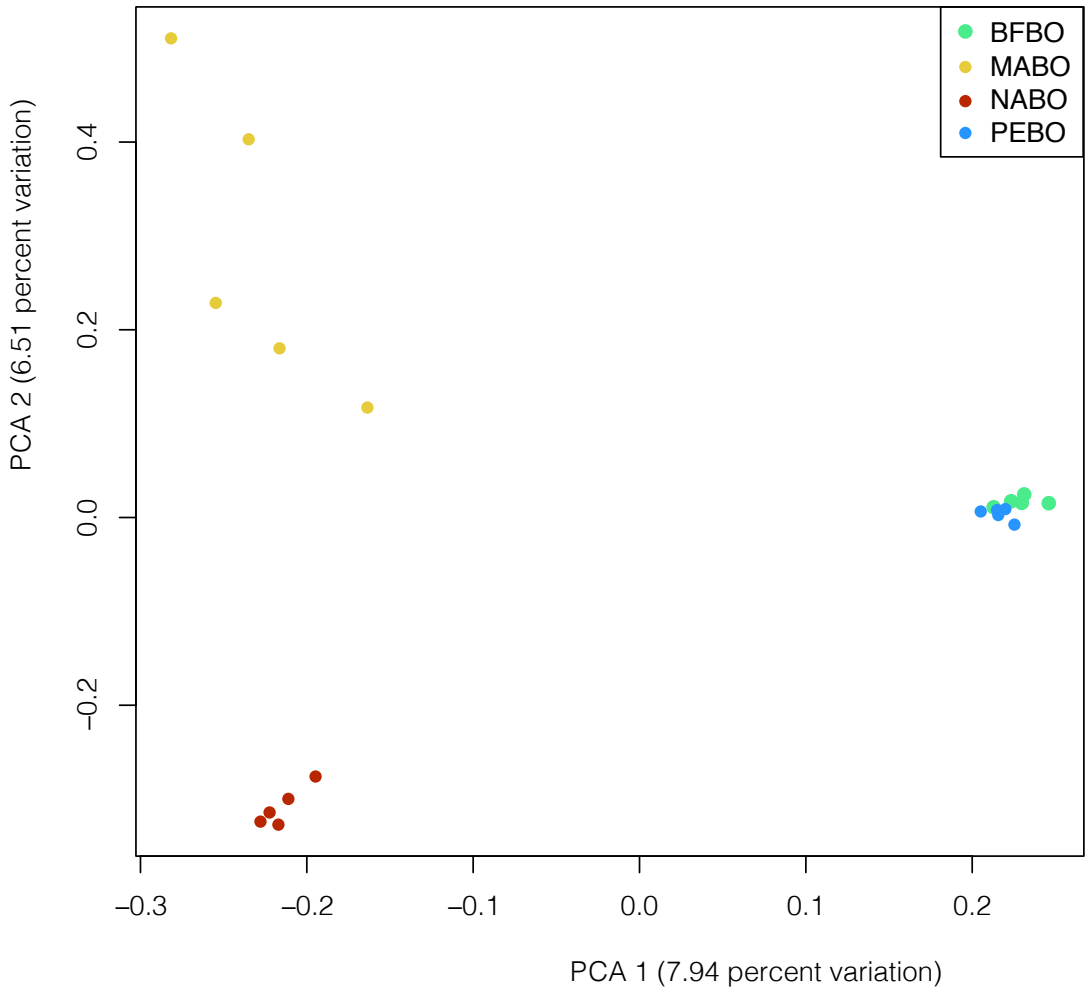


Figure S3. Principal component analysis of the sister species masked (MABO) and Nazca (NABO) boobies.

Principal component 1 separated the samples by species, and principal component 2 separated the masked booby samples by ocean basin (PC1 = 14.72 percent variation, PC2 = 12.25 percent variation).

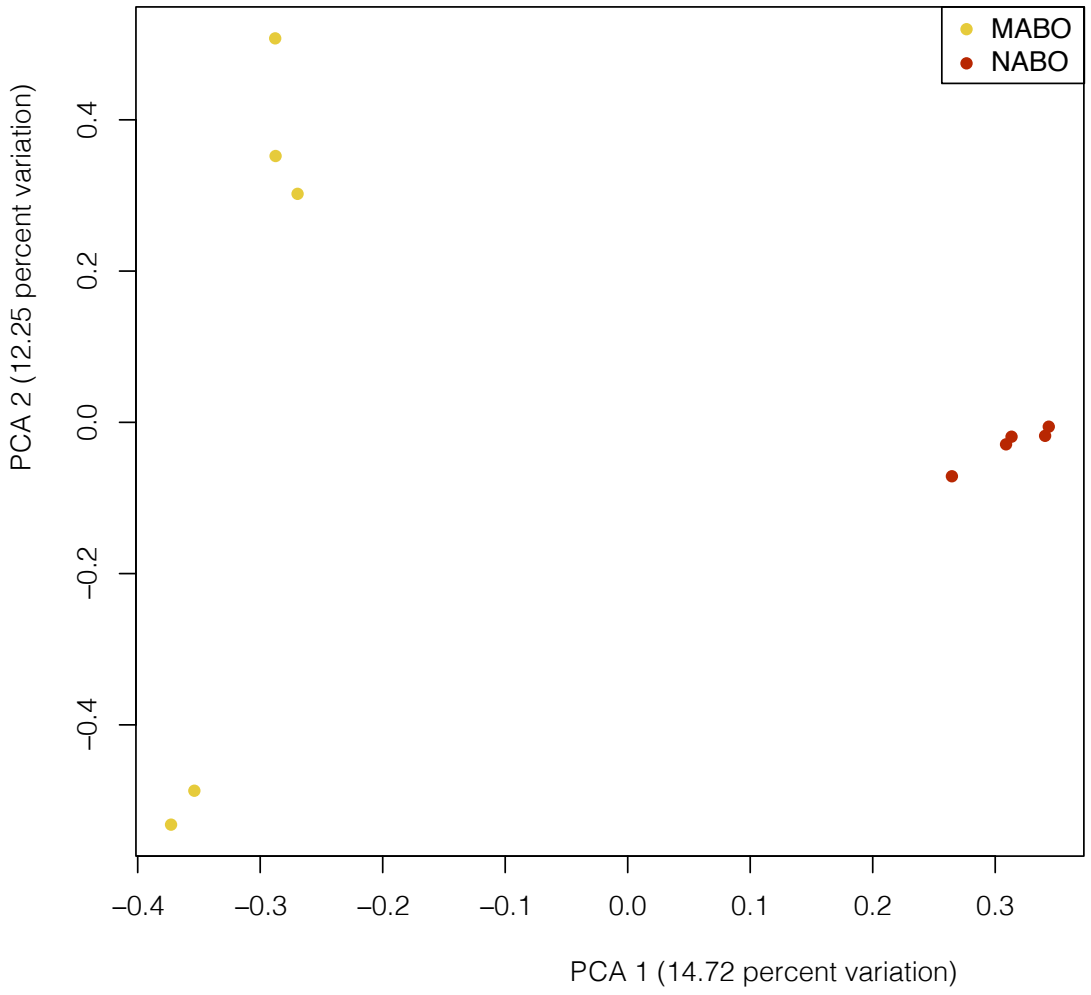


Figure S4. Principal component analysis of the sister species blue-footed (BFBO) and Peruvian (PEBO) boobies.

Principal component 1 separated the samples out by species, and no geographic pattern is discernible in PC2 (PC1 = 13.68 percent variation, PC2 = 11.03 percent variation).

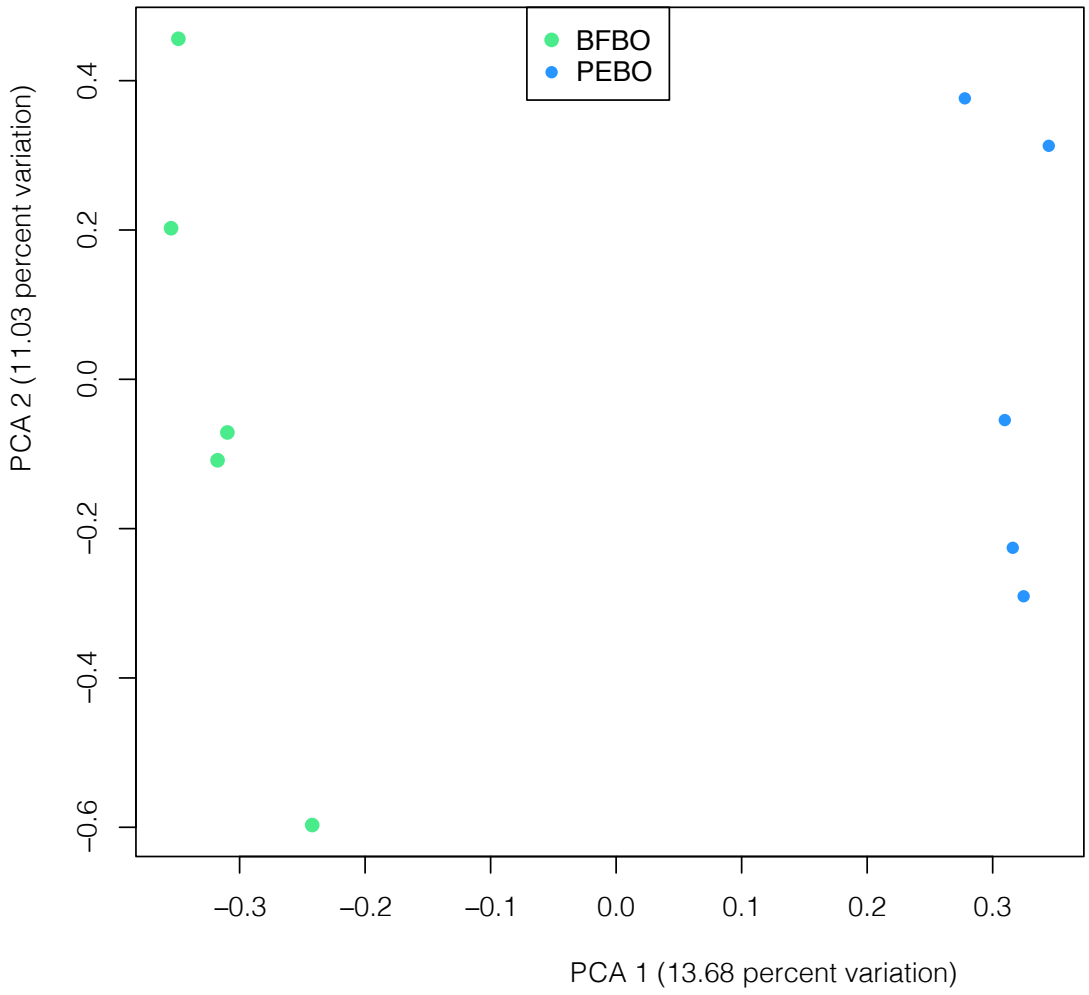


Figure S5. Principal component analysis of red-footed booby (RFBO) samples.

Principal component 1 separated the Caribbean Sea and Indian Ocean samples from the Pacific Ocean samples (PC1 = 20.91 percent variation, PC2 = 20.33 percent variation).

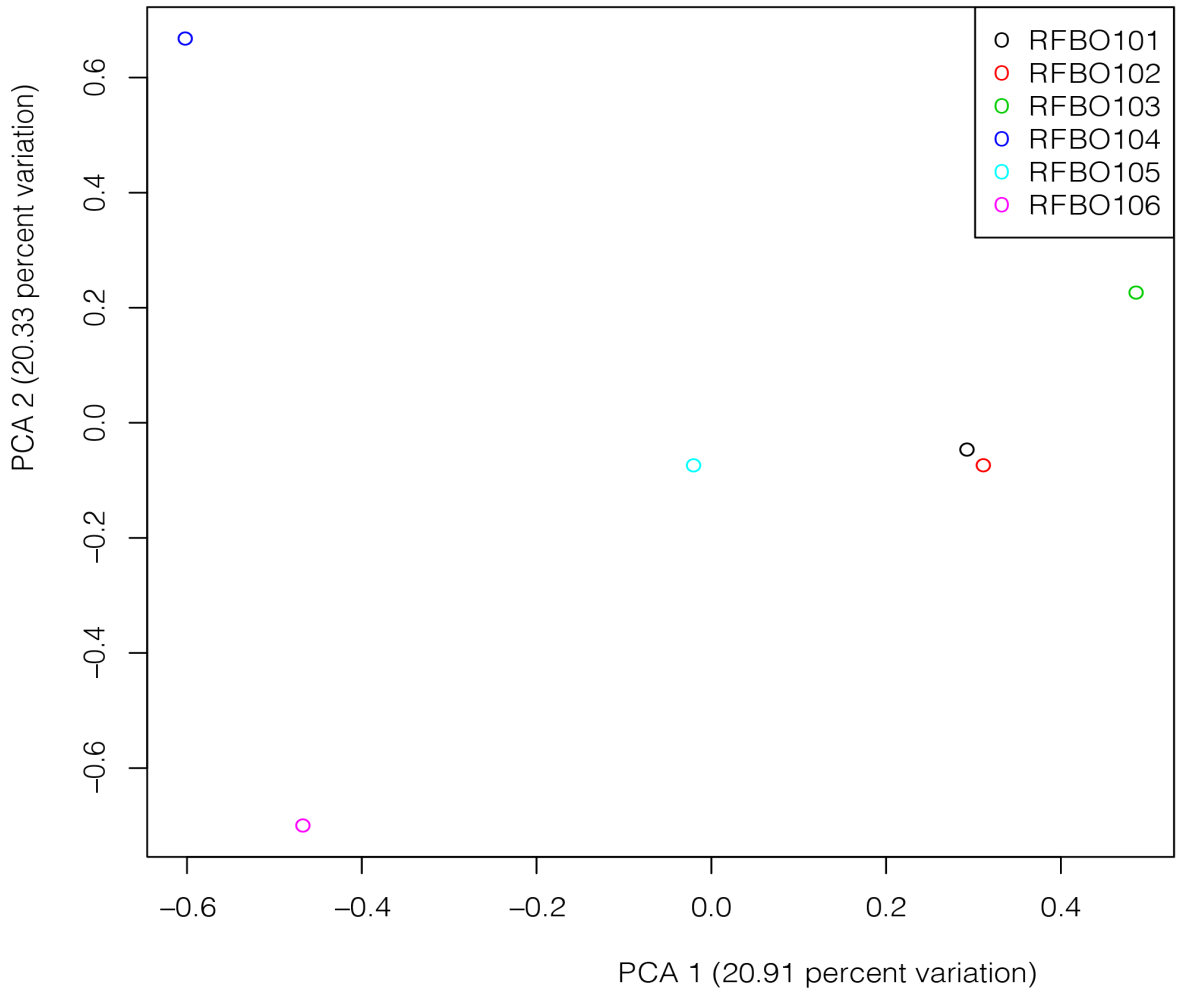


Figure S6. Principal component analysis of brown booby (BRBO) samples.

Principal component 1 separated the Caribbean Sea and Atlantic Ocean samples from the Pacific Ocean samples (PC1 = 38.30 percent variation, PCA2 = 31.98 percent variation).

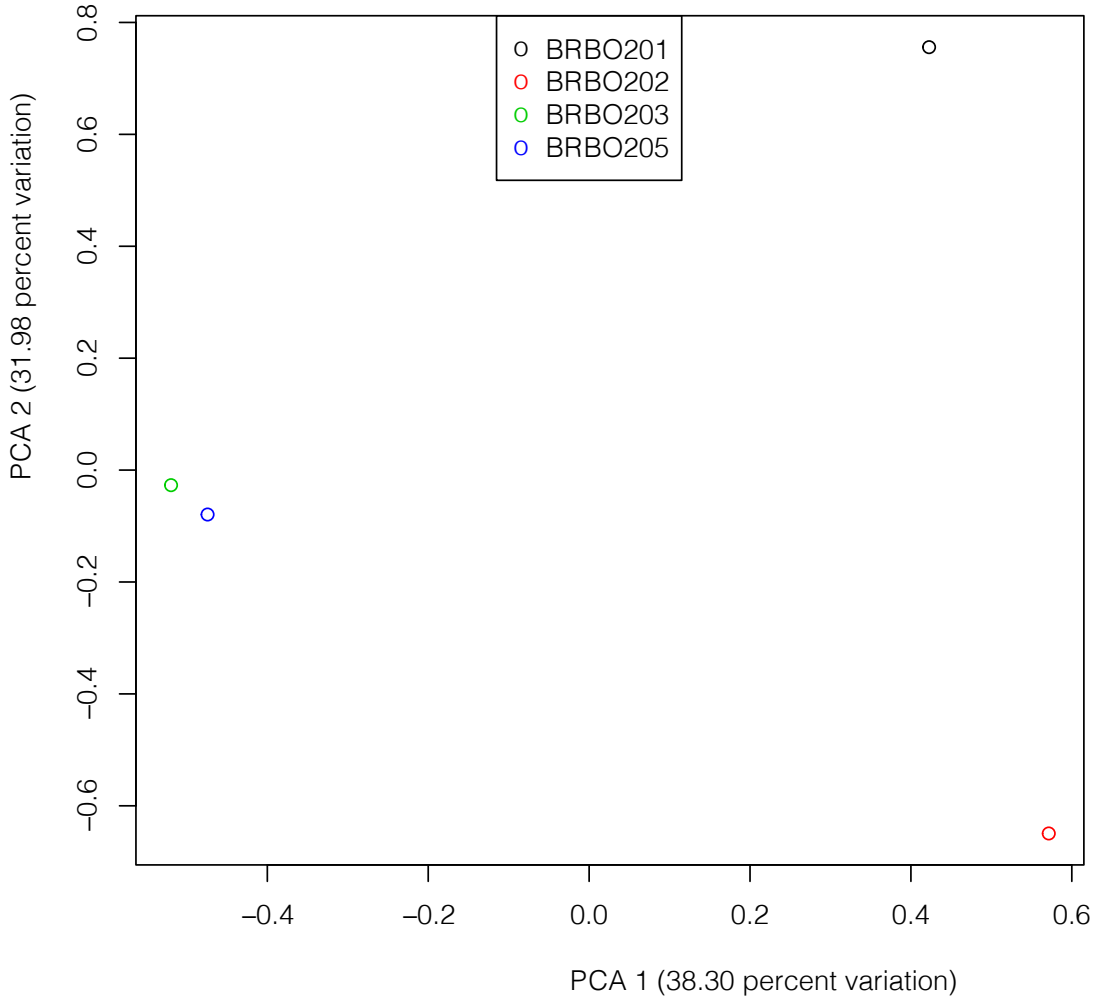


Figure S7. Principal component analysis of masked booby (MABO) samples.

Principal component 1 separated the samples based on assignment to either the Pacific and Indian Oceans or the Caribbean Sea and Atlantic Ocean (PC1 = 29.73 percent variation, PCA2 = 24.01 percent variation).

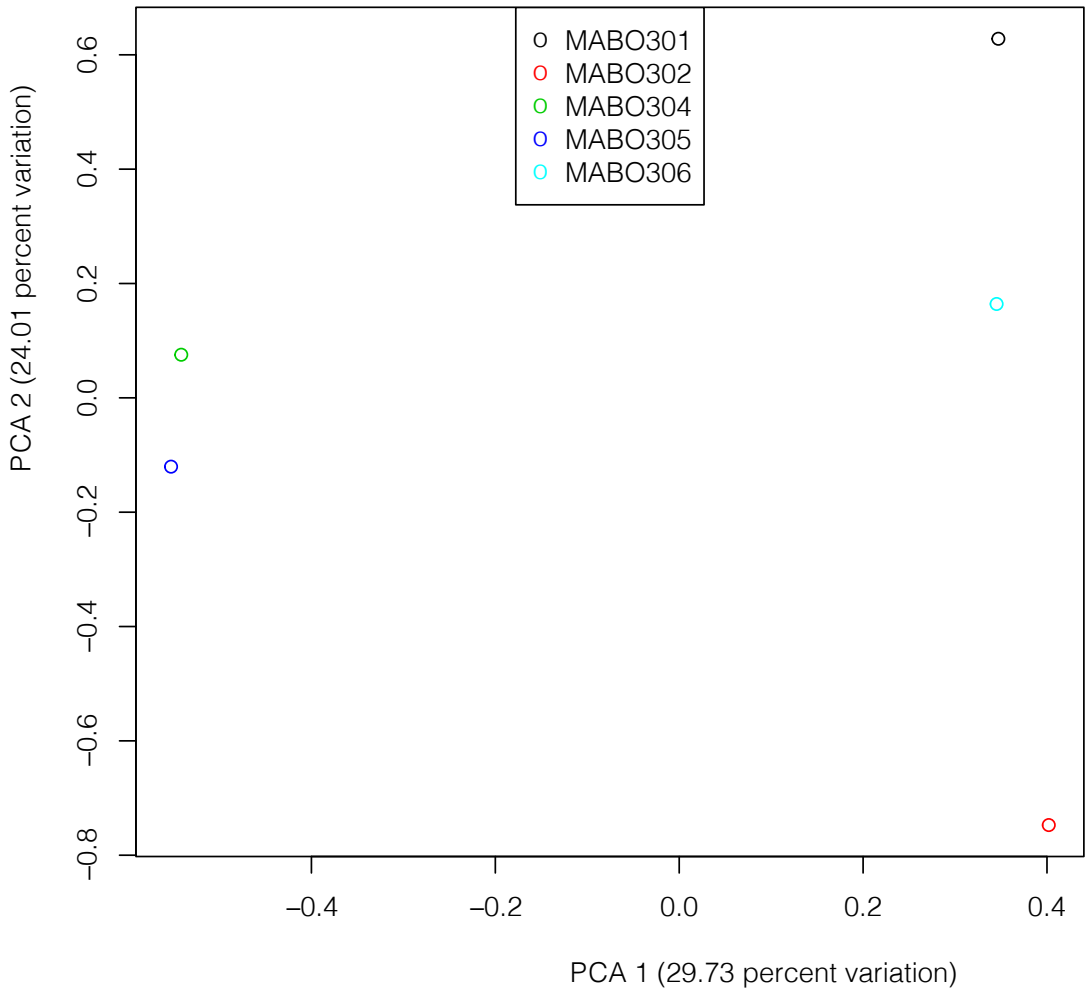


Figure S8. Principal component analysis of Nazca booby (NABO) samples.

No geographic patterns were discernible (PC1 = 25.92 percent variation, PC2 = 27.90 percent variation).

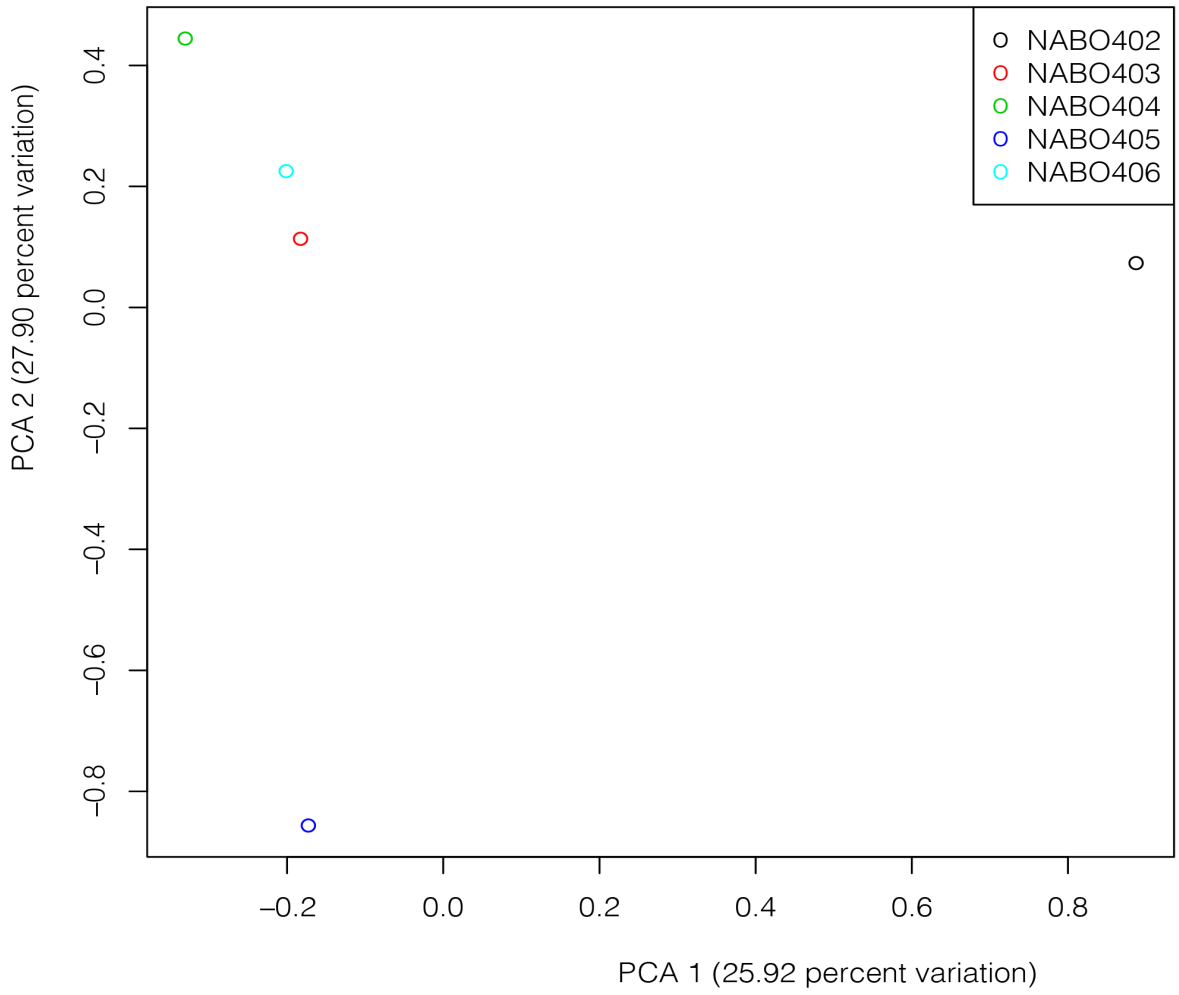


Figure S9. Principal component analysis of Peruvian booby (PEBO) samples.

Principal component 1 separated the two southernmost Peruvian booby samples from all other samples (PC1 = 25.49 percent variation, PCA2 = 24.96 percent variation).

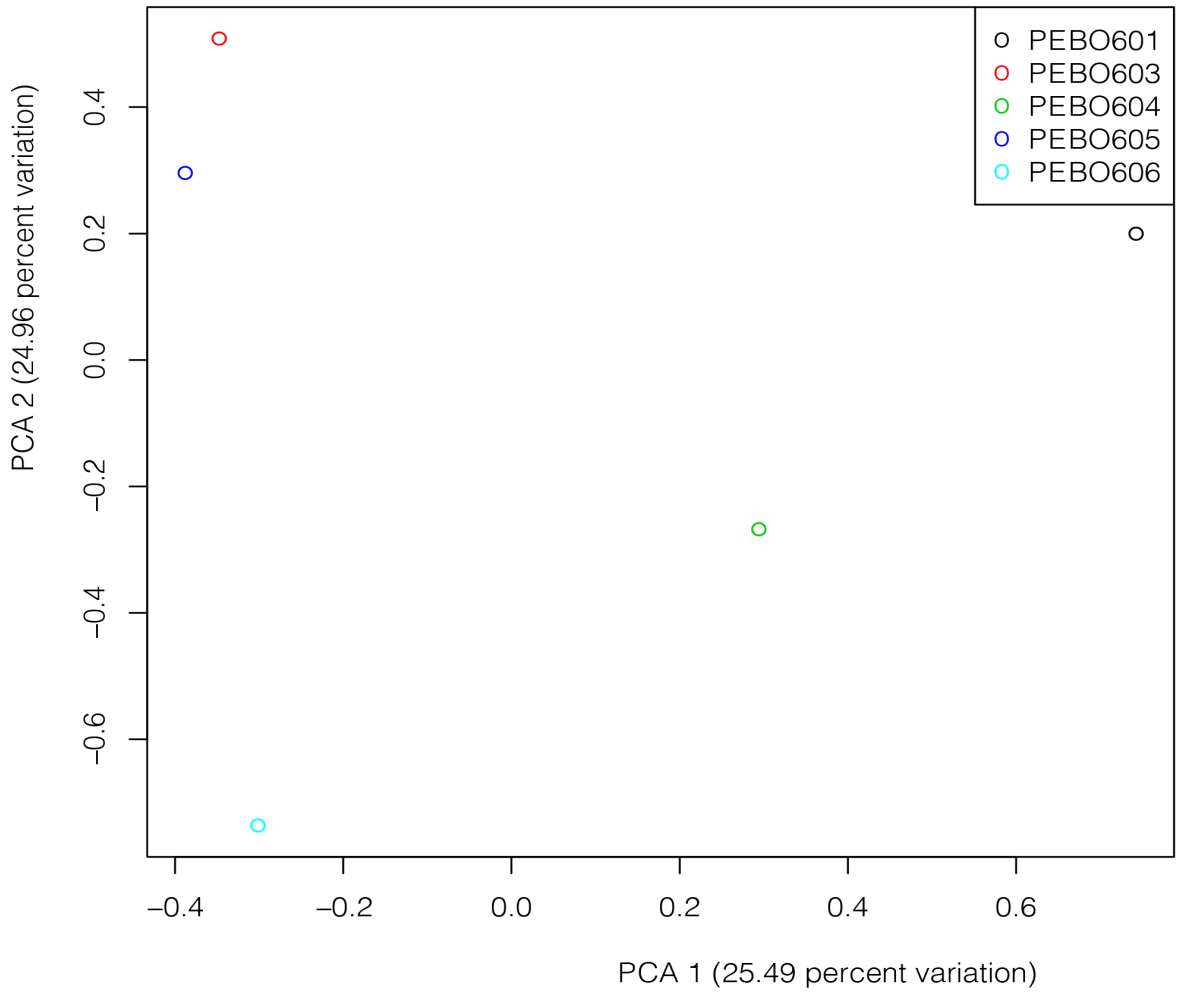


Figure S10. Principal component analysis of blue-footed booby (BFBO) samples.

Principal component 1 separated the northernmost blue-footed booby sample from all other samples (PC1 = 25.86 percent variation, PCA2 = 25.06 percent variation).

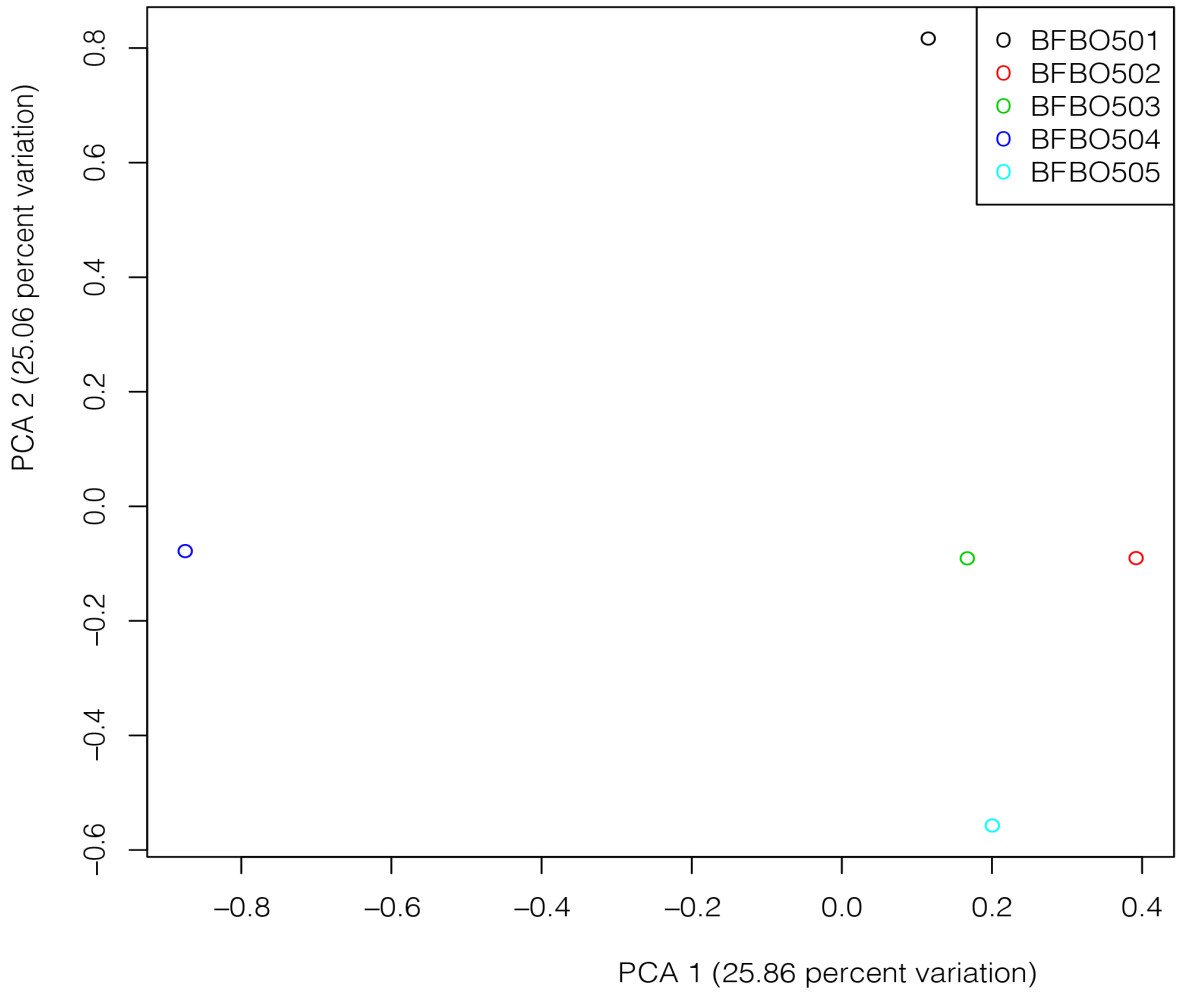


Figure S11. Species level phylogenetic network modeled in phylonet.

This model had the best probability, AIC, AICc, and BIC scores of the models that we constructed. Total log probability = -8941195.9, AIC = 17882427.7, AICc = 17882427.83, and BIC = 17882547.36.

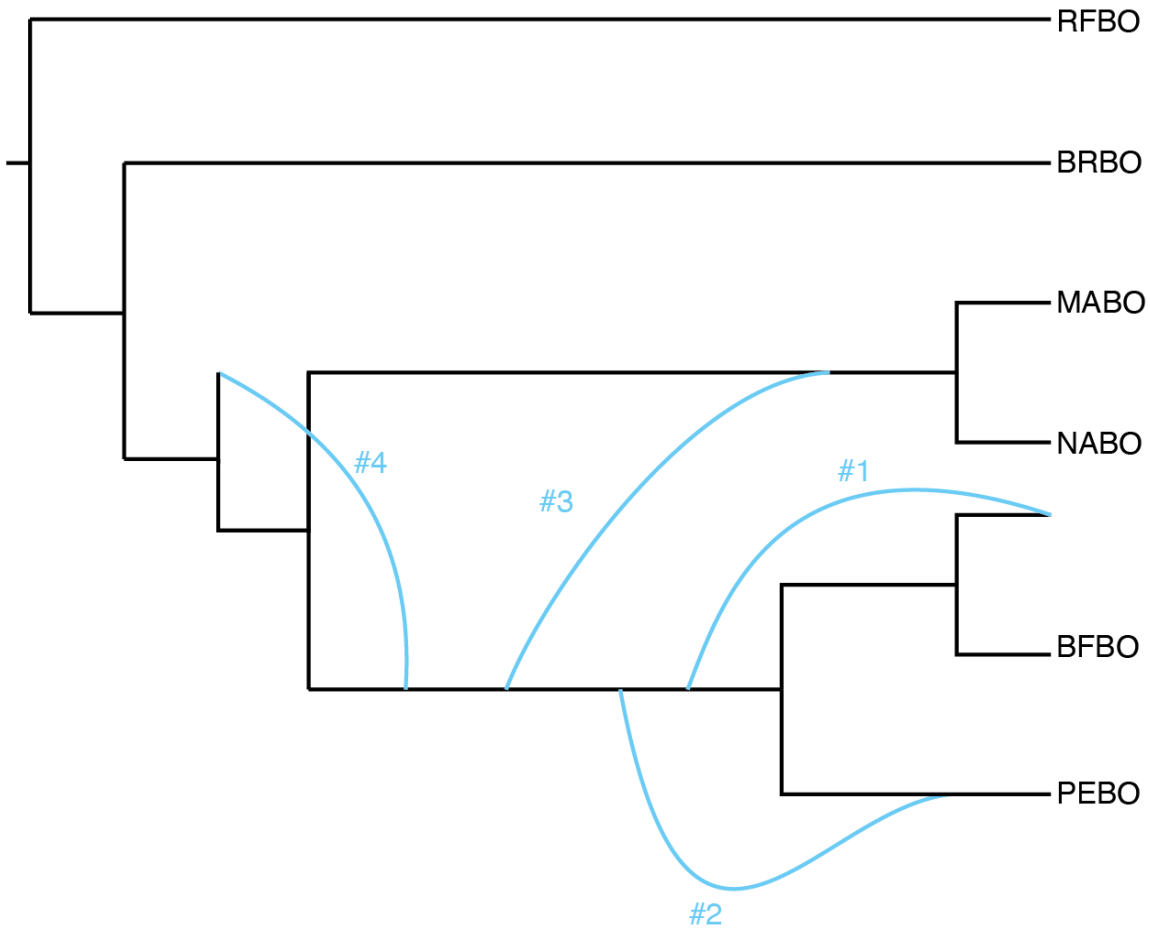
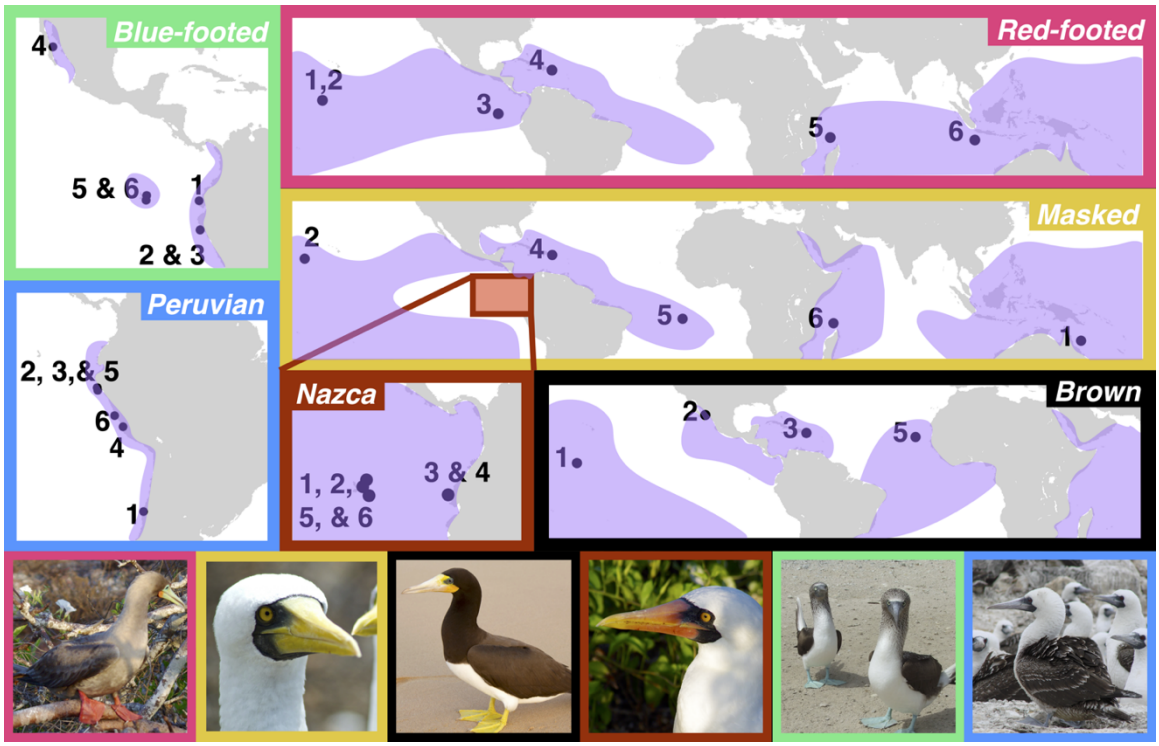


Figure S12. Species distributions maps.

Distributions of species shown in purple. Species mainly breed on islands within these broad ranges, and all ranges are estimations. Maps were produced in Illustrator based on maps found in Birds of the World (Carboneras 2020, Cuccaro Diaz et al. 2020, Grace et al. 2020, Hernández Díaz and Salazar Gómez, 2020, Schreiber et al. 2020a, Schreiber et al. 2020b). We only show broad distribution, but more detailed depictions of the breeding distributions can be found on Birds of the World or in Nelson 1978.



Tables

Table S1: Tests for Introgression (intraspecies)

Results of ABBA BABA tests for shared genetic variation between populations within each red-footed boobies, brown boobies, and masked boobies. Only the results presented in Figure 3 are presented here. A Z score above |2.58| is considered significant here. Tests with significant values are bolded.

Test	Species	P1	P2	P3	O	D	D sd	D Z
5a	**Red-footed**	**1**	**5**	**6**	**Masked**	**0.5982**	**0.1409**	**191.338**
5b	**Red-footed**	**3**	**5**	**6**	**Masked**	**0.0705**	**0.2434**	**13.059**
5c	**Red-footed**	**1**	**3**	**6**	**Masked**	**0.6111**	**0.0687**	**401.043**
5d	**Red-footed**	**1**	**3**	**5**	**Masked**	**0.6118**	**0.0662**	**416.419**
6a	**Brown**	**2**	**1**	**35**	**Red-footed**	**0.0154**	**0.2384**	**2.659**
6b	**Brown**	**5**	**3**	**12**	**Red-footed**	**0.0598**	**0.202**	**12.885**
7a	**Masked**	**1**	**6**	**45**	**Red-footed**	**0.233**	**0.1453**	**69.423**
7b	**Masked**	**1,6**	**2**	**45**	**Red-footed**	**0.3234**	**0.18**	**78.439**
7c	**Masked**	**5**	**4**	**162**	**Red-footed**	**0.3234**	**0.18**	**78.439**

Table S2: AIC Calculation

reticulation_edges	Run	Inferred Tree	number_taxa	number_reticulations	K	Ln(L)	AIC
1	1	1	6	1	12	-8970852.1	17941728.2
1	1	2	6	1	12	-8983174.1	17966372.2
1	1	3	6	1	12	-8983179.6	17966383.2
1	1	4	6	1	12	-8983179.6	17966383.2
1	1	5	6	1	12	-8983183.8	17966391.6
1	2	1	6	1	12	-8970877.2	17941778.5
1	2	2	6	1	12	-8983173.7	17966371.3
1	2	3	6	1	12	-8983175.7	17966375.4
1	2	4	6	1	12	-8983180	17966383.9
1	2	5	6	1	12	-8983183.4	17966390.9
1	3	1	6	1	12	-8983173.4	17966370.9
1	3	2	6	1	12	-8983174.2	17966372.5
1	3	3	6	1	12	-8983174.3	17966372.6
1	3	4	6	1	12	-8983175.6	17966375.2
1	3	5	6	1	12	-8983179.1	17966382.2
2	1	1	6	2	14	-8982454.2	17964936.4
2	1	2	6	2	14	-8983175.7	17966379.4
2	1	3	6	2	14	-8983184.1	17966396.1
2	1	4	6	2	14	-8983189	17966406.1
2	1	5	6	2	14	-8983189.2	17966406.4
2	2	1	6	2	14	-8970871.7	17941771.4
2	2	2	6	2	14	-8981390.6	17962809.2
2	2	3	6	2	14	-8983174	17966376
2	2	4	6	2	14	-8983175.7	17966379.4
2	2	5	6	2	14	-8983180.5	17966388.9
2	3	1	6	2	14	-8982468.2	17964964.4
2	3	2	6	2	14	-8983178.5	17966385
2	3	3	6	2	14	-8983178.8	17966385.5
2	3	4	6	2	14	-8983179	17966386
2	3	5	6	2	14	-8983183.1	17966394.1
3	1	1	6	3	16	-8966351.3	17932734.6
3	1	2	6	3	16	-8970876.2	17941784.5
3	1	3	6	3	16	-8971891.2	17943814.4
3	1	4	6	3	16	-8973324.6	17946681.1
3	1	5	6	3	16	-8979706.5	17959445.1
3	2	1	6	3	16	-8944215.5	17888463.1

3	2	2	6	3	16	-8983174.2	17966380.5
3	2	3	6	3	16	-8983183	17966398.1
3	2	4	6	3	16	-8983183	17966398.1
3	2	5	6	3	16	-8983183	17966398.1
3	3	1	6	3	16	-8970862.8	17941757.5
3	3	2	6	3	16	-8970875.2	17941782.4
3	3	3	6	3	16	-8981086.6	17962205.2
3	3	4	6	3	16	-8983173.4	17966378.8
3	3	5	6	3	16	-8983173.8	17966379.6
4	1	1	6	4	18	-8943022.9	17886081.9
4	1	2	6	4	18	-8943123.3	17886282.7
4	1	3	6	4	18	-8945962	17891960.1
4	1	4	6	4	18	-8961684.4	17923404.8
4	1	5	6	4	18	-8970801.3	17941638.6
4	2	1	6	4	18	-8980845.9	17961727.7
4	2	2	6	4	18	-8981285.7	17962607.3
4	2	3	6	4	18	-8983179.9	17966395.7
4	2	4	6	4	18	-8983183	17966402.1
4	2	5	6	4	18	-8983186.3	17966408.5
4	3	1	6	4	18	-8941195.9	17882427.7
4	3	2	6	4	18	-8963005.3	17926046.5
4	3	3	6	4	18	-8978908.4	17957852.8
4	3	4	6	4	18	-8983183.1	17966402.2
4	3	5	6	4	18	-8983190.2	17966416.3

Table S3: AICc Calculation

reticulation_edges	Run	Inferred Tree	number_taxa	number_reticulations	K	Ln(L)	n_Data points	n/n-K-1	AICc
1	1	1	6	1	12	-8970852.1	5697	1.00228712	17941728.3
1	1	2	6	1	12	-8983174.1	5697	1.00228712	17966372.3
1	1	3	6	1	12	-8983179.6	5697	1.00228712	17966383.2
1	1	4	6	1	12	-8983179.6	5697	1.00228712	17966383.2
1	1	5	6	1	12	-8983183.8	5697	1.00228712	17966391.7
1	2	1	6	1	12	-8970877.2	5697	1.00228712	17941778.5
1	2	2	6	1	12	-8983173.7	5697	1.00228712	17966371.4
1	2	3	6	1	12	-8983175.7	5697	1.00228712	17966375.4
1	2	4	6	1	12	-8983180	5697	1.00228712	17966384
1	2	5	6	1	12	-8983183.4	5697	1.00228712	17966390.9
1	3	1	6	1	12	-8983173.4	5697	1.00228712	17966370.9
1	3	2	6	1	12	-8983174.2	5697	1.00228712	17966372.5
1	3	3	6	1	12	-8983174.3	5697	1.00228712	17966372.7
1	3	4	6	1	12	-8983175.6	5697	1.00228712	17966375.3
1	3	5	6	1	12	-8983179.1	5697	1.00228712	17966382.3
2	1	1	6	2	14	-8982454.2	5697	1.00263992	17964936.5
2	1	2	6	2	14	-8983175.7	5697	1.00263992	17966379.5
2	1	3	6	2	14	-8983184.1	5697	1.00263992	17966396.2
2	1	4	6	2	14	-8983189	5697	1.00263992	17966406.1
2	1	5	6	2	14	-8983189.2	5697	1.00263992	17966406.5
2	2	1	6	2	14	-8970871.7	5697	1.00263992	17941771.4
2	2	2	6	2	14	-8981390.6	5697	1.00263992	17962809.3
2	2	3	6	2	14	-8983174	5697	1.00263992	17966376
2	2	4	6	2	14	-8983175.7	5697	1.00263992	17966379.5
2	2	5	6	2	14	-8983180.5	5697	1.00263992	17966389
2	3	1	6	2	14	-8982468.2	5697	1.00263992	17964964.5
2	3	2	6	2	14	-8983178.5	5697	1.00263992	17966385.1
2	3	3	6	2	14	-8983178.8	5697	1.00263992	17966385.6
2	3	4	6	2	14	-8983179	5697	1.00263992	17966386.1
2	3	5	6	2	14	-8983183.1	5697	1.00263992	17966394.2
3	1	1	6	3	16	-8966351.3	5697	1.00299296	17932734.7
3	1	2	6	3	16	-8970876.2	5697	1.00299296	17941784.6
3	1	3	6	3	16	-8971891.2	5697	1.00299296	17943814.5
3	1	4	6	3	16	-8973324.6	5697	1.00299296	17946681.2
3	1	5	6	3	16	-8979706.5	5697	1.00299296	17959445.2

3	2	1	6	3	16	-8944215.5	5697	1.00299296	17888463.2
3	2	2	6	3	16	-8983174.2	5697	1.00299296	17966380.6
3	2	3	6	3	16	-8983183	5697	1.00299296	17966398.2
3	2	4	6	3	16	-8983183	5697	1.00299296	17966398.2
3	2	5	6	3	16	-8983183	5697	1.00299296	17966398.2
3	3	1	6	3	16	-8970862.8	5697	1.00299296	17941757.6
3	3	2	6	3	16	-8970875.2	5697	1.00299296	17941782.5
3	3	3	6	3	16	-8981086.6	5697	1.00299296	17962205.3
3	3	4	6	3	16	-8983173.4	5697	1.00299296	17966378.9
3	3	5	6	3	16	-8983173.8	5697	1.00299296	17966379.7
4	1	1	6	4	18	-8943022.9	5697	1.00334625	17886082
4	1	2	6	4	18	-8943123.3	5697	1.00334625	17886282.8
4	1	3	6	4	18	-8945962	5697	1.00334625	17891960.2
4	1	4	6	4	18	-8961684.4	5697	1.00334625	17923404.9
4	1	5	6	4	18	-8970801.3	5697	1.00334625	17941638.8
4	2	1	6	4	18	-8980845.9	5697	1.00334625	17961727.8
4	2	2	6	4	18	-8981285.7	5697	1.00334625	17962607.4
4	2	3	6	4	18	-8983179.9	5697	1.00334625	17966395.8
4	2	4	6	4	18	-8983183	5697	1.00334625	17966402.2
4	2	5	6	4	18	-8983186.3	5697	1.00334625	17966408.6
4	3	1	6	4	18	-8941195.9	5697	1.00334625	17882427.8
4	3	2	6	4	18	-8963005.3	5697	1.00334625	17926046.7
4	3	3	6	4	18	-8978908.4	5697	1.00334625	17957852.9
4	3	4	6	4	18	-8983183.1	5697	1.00334625	17966402.3
4	3	5	6	4	18	-8983190.2	5697	1.00334625	17966416.4

Table S4: BIC Calculation

reticulation_edges	Run	Inferred Tree	number_taxa	number_reticulations	K	Ln(L)	n_Data points	BIC
1	1	1	6	1	12	-8970852.1	5697	17941808
1	1	2	6	1	12	-8983174.1	5697	17966452
1	1	3	6	1	12	-8983179.6	5697	17966462.9
1	1	4	6	1	12	-8983179.6	5697	17966462.9
1	1	5	6	1	12	-8983183.8	5697	17966471.4
1	2	1	6	1	12	-8970877.2	5697	17941858.2
1	2	2	6	1	12	-8983173.7	5697	17966451.1
1	2	3	6	1	12	-8983175.7	5697	17966455.1
1	2	4	6	1	12	-8983180	5697	17966463.7
1	2	5	6	1	12	-8983183.4	5697	17966470.6
1	3	1	6	1	12	-8983173.4	5697	17966450.6
1	3	2	6	1	12	-8983174.2	5697	17966452.3
1	3	3	6	1	12	-8983174.3	5697	17966452.4
1	3	4	6	1	12	-8983175.6	5697	17966455
1	3	5	6	1	12	-8983179.1	5697	17966462
2	1	1	6	2	14	-8982454.2	5697	17965029.5
2	1	2	6	2	14	-8983175.7	5697	17966472.5
2	1	3	6	2	14	-8983184.1	5697	17966489.2
2	1	4	6	2	14	-8983189	5697	17966499.1
2	1	5	6	2	14	-8983189.2	5697	17966499.4
2	2	1	6	2	14	-8970871.7	5697	17941864.4
2	2	2	6	2	14	-8981390.6	5697	17962902.3
2	2	3	6	2	14	-8983174	5697	17966469
2	2	4	6	2	14	-8983175.7	5697	17966472.5
2	2	5	6	2	14	-8983180.5	5697	17966482
2	3	1	6	2	14	-8982468.2	5697	17965057.4
2	3	2	6	2	14	-8983178.5	5697	17966478.1
2	3	3	6	2	14	-8983178.8	5697	17966478.6
2	3	4	6	2	14	-8983179	5697	17966479.1
2	3	5	6	2	14	-8983183.1	5697	17966487.2
3	1	1	6	3	16	-8966351.3	5697	17932841
3	1	2	6	3	16	-8970876.2	5697	17941890.8
3	1	3	6	3	16	-8971891.2	5697	17943920.7
3	1	4	6	3	16	-8973324.6	5697	17946787.5
3	1	5	6	3	16	-8979706.5	5697	17959551.4

3	2	1	6	3	16	-8944215.5	5697	17888569.5
3	2	2	6	3	16	-8983174.2	5697	17966486.9
3	2	3	6	3	16	-8983183	5697	17966504.4
3	2	4	6	3	16	-8983183	5697	17966504.4
3	2	5	6	3	16	-8983183	5697	17966504.4
3	3	1	6	3	16	-8970862.8	5697	17941863.9
3	3	2	6	3	16	-8970875.2	5697	17941888.8
3	3	3	6	3	16	-8981086.6	5697	17962311.6
3	3	4	6	3	16	-8983173.4	5697	17966485.1
3	3	5	6	3	16	-8983173.8	5697	17966486
4	1	1	6	4	18	-8943022.9	5697	17886201.5
4	1	2	6	4	18	-8943123.3	5697	17886402.3
4	1	3	6	4	18	-8945962	5697	17892079.7
4	1	4	6	4	18	-8961684.4	5697	17923524.5
4	1	5	6	4	18	-8970801.3	5697	17941758.3
4	2	1	6	4	18	-8980845.9	5697	17961847.4
4	2	2	6	4	18	-8981285.7	5697	17962727
4	2	3	6	4	18	-8983179.9	5697	17966515.4
4	2	4	6	4	18	-8983183	5697	17966521.7
4	2	5	6	4	18	-8983186.3	5697	17966528.2
4	3	1	6	4	18	-8941195.9	5697	17882547.4
4	3	2	6	4	18	-8963005.3	5697	17926166.2
4	3	3	6	4	18	-8978908.4	5697	17957972.5
4	3	4	6	4	18	-8983183.1	5697	17966521.9
4	3	5	6	4	18	-8983190.2	5697	17966536

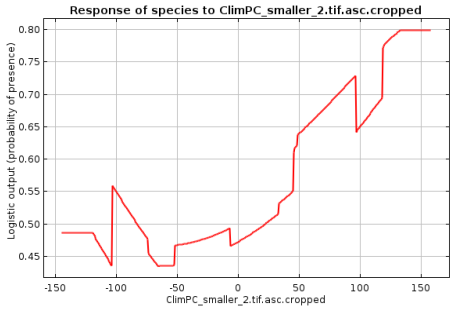
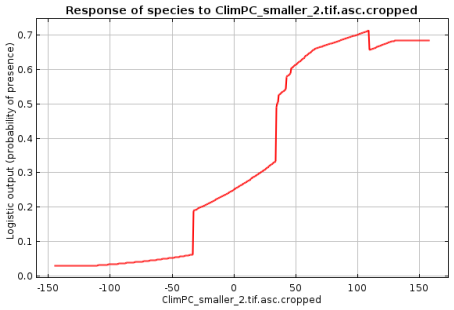
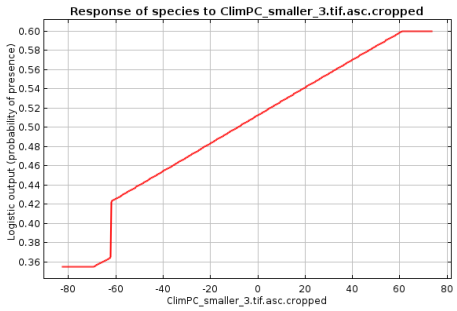
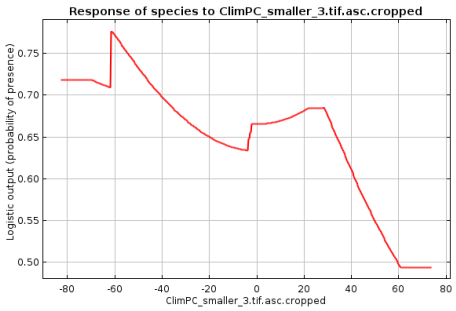
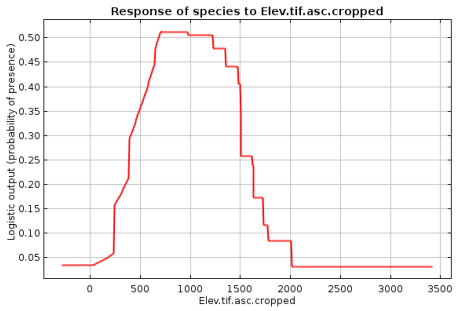
APPENDIX B

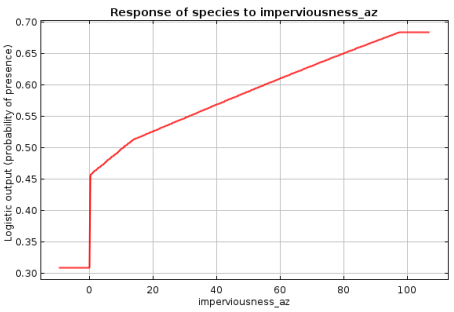
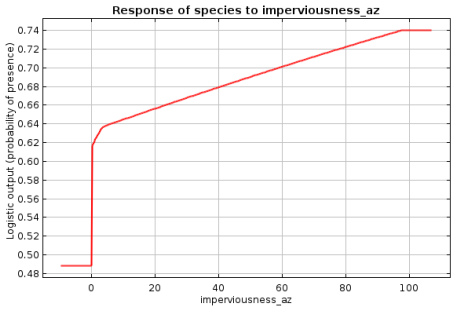
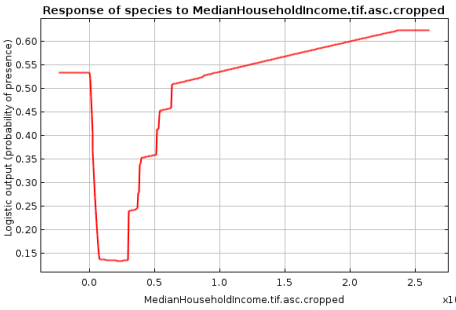
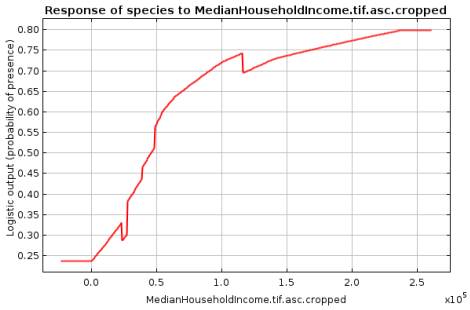
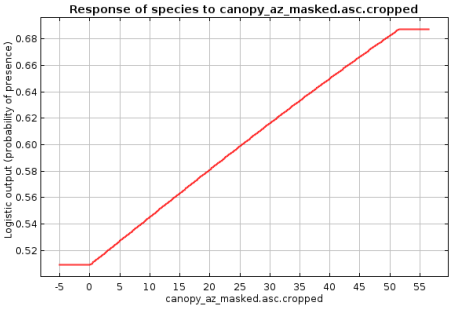
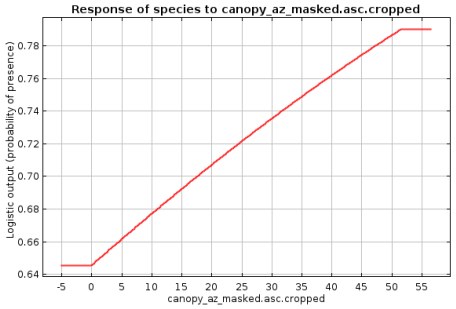
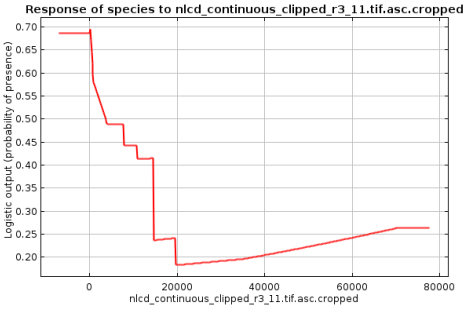
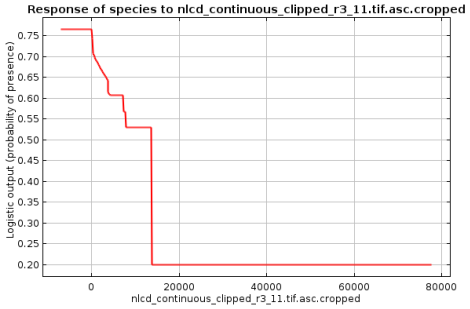
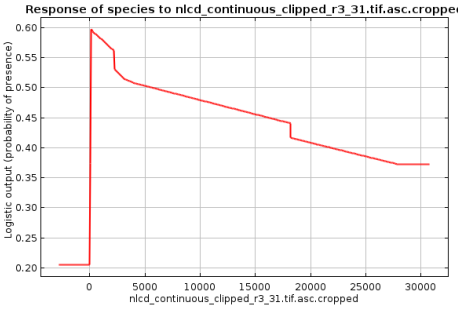
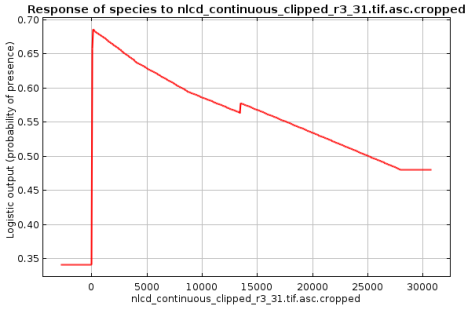
CHAPTER 2 SUPPLEMENTARY TABLES AND FIGURES

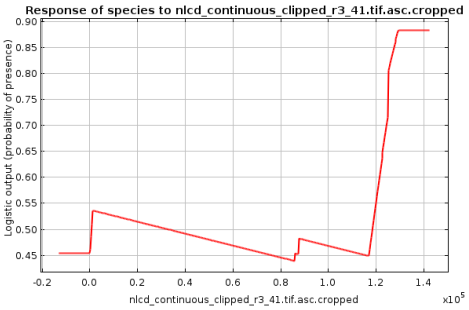
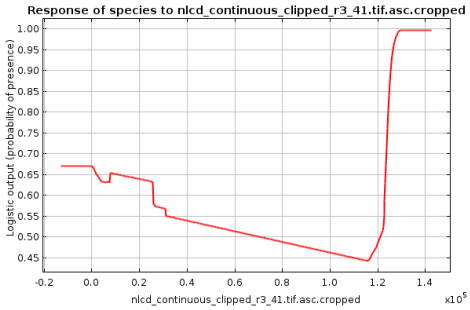
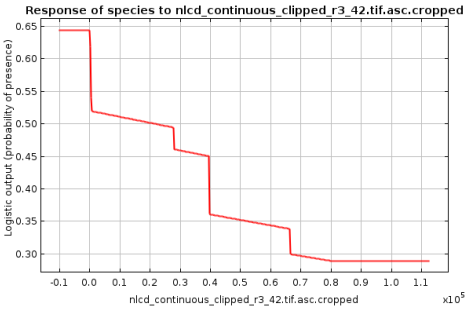
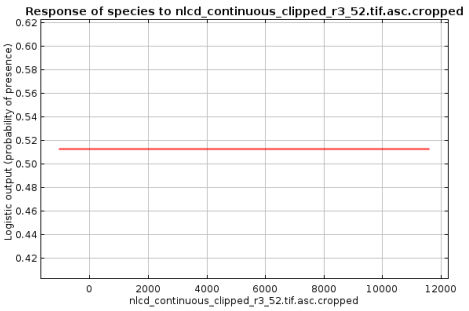
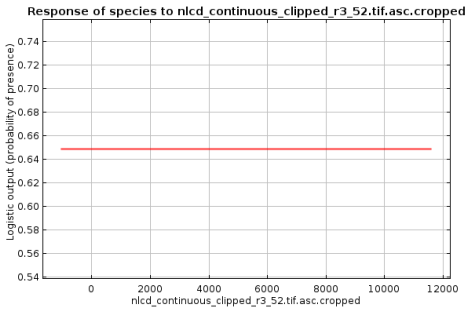
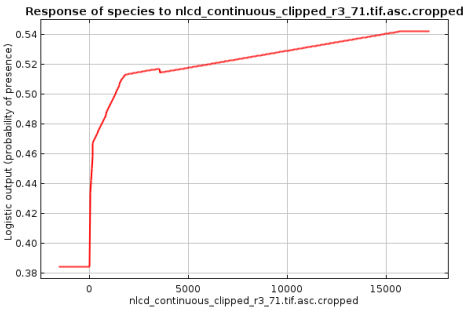
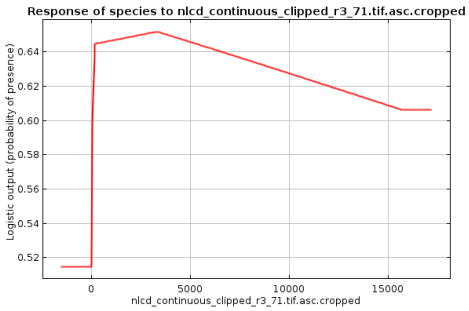
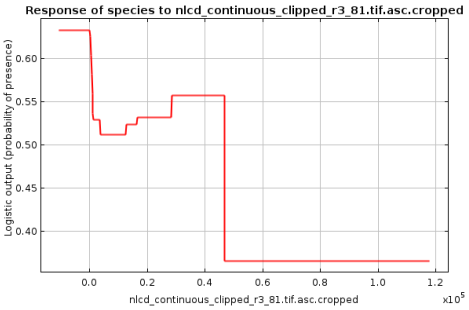
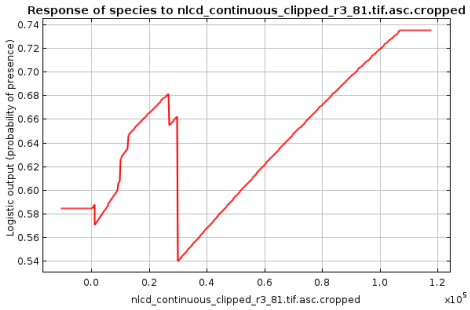
Figures

Figure S1: Marginal Response of Species to Environmental Variables across Arizona

These plots are produced by the Maxent software and depict how each environmental variable affects the maxent prediction of occurrence of the species across the range. These plots display predicted probability of occurrence on the y-axis with all other variables set to their average value.

Variable	Northern Cardinal	Pyrrhuloxia
Climate PC2		
Climate PC3		
Elevation		

% Surface Imperviousness		
Median Household Income		
Canopy		
Distance from Open Water		
Distance from Barren Land		

Distance from Deciduous Forest		
Distance from Evergreen Forest		
Distance from Shrub/Scrub		
Distance from Grassland/ Herbaceous		
Distance from Pasture/Hay		

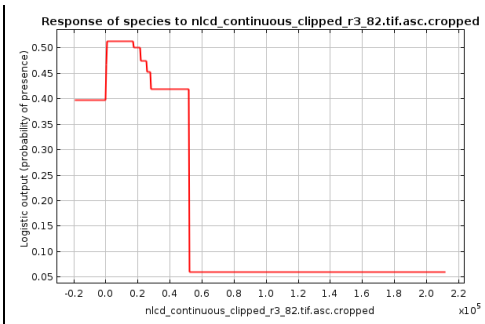
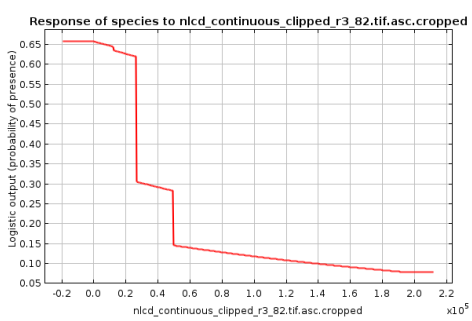
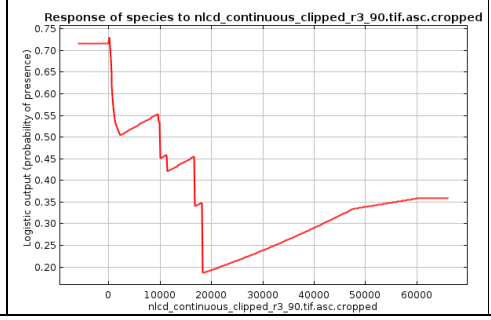
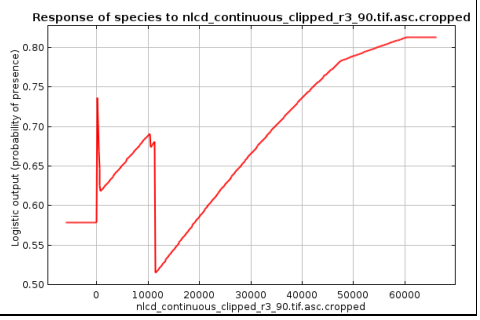
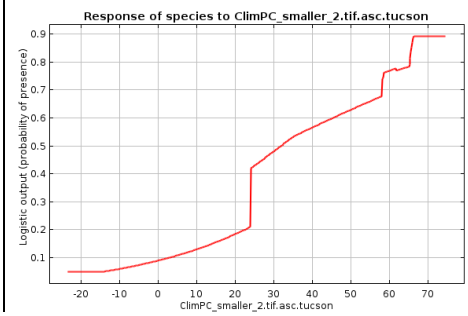
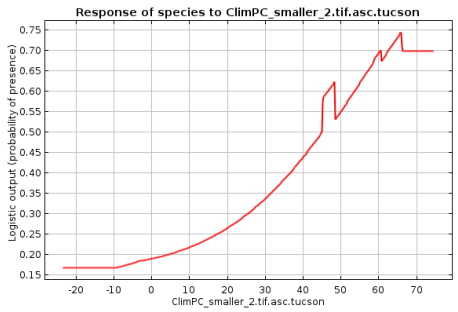
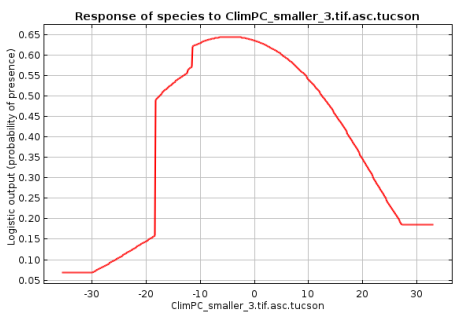
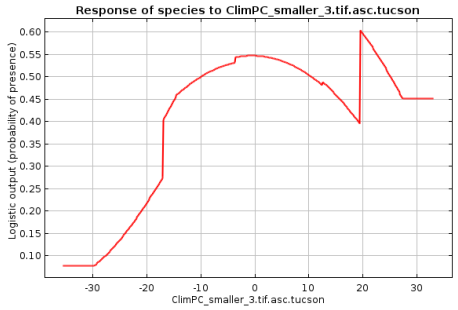
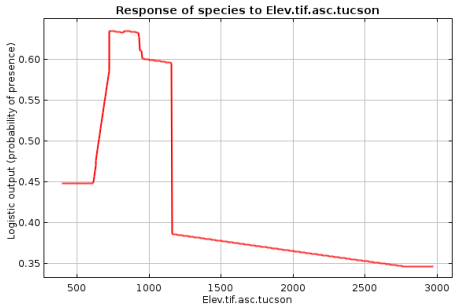
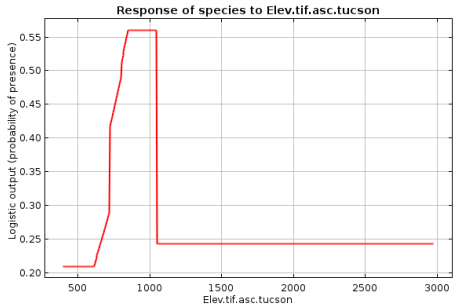
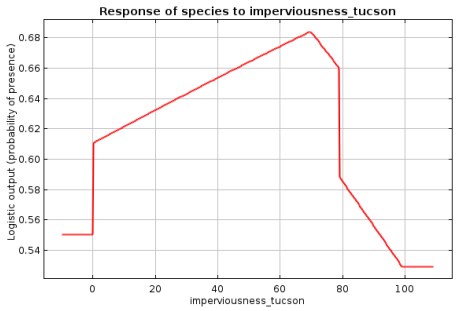
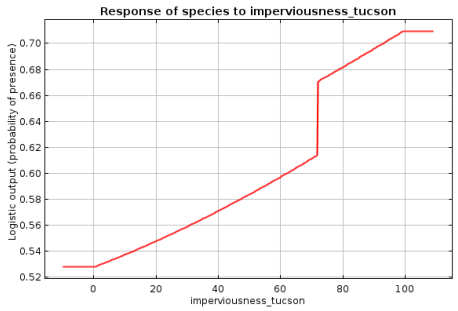
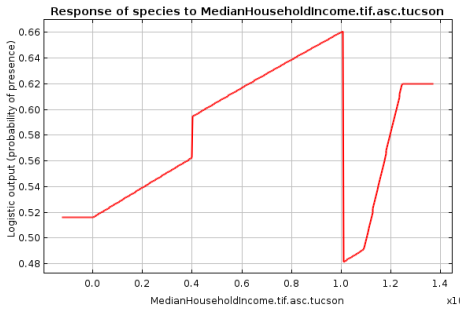
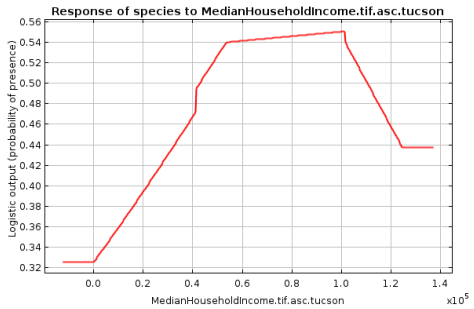
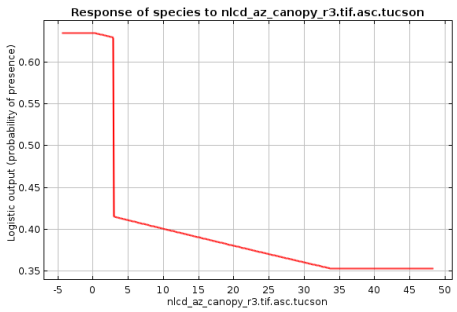
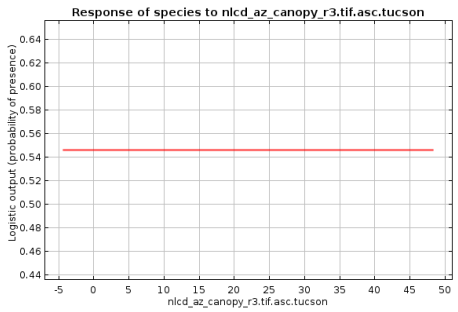
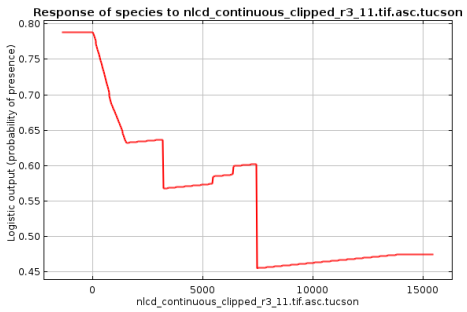
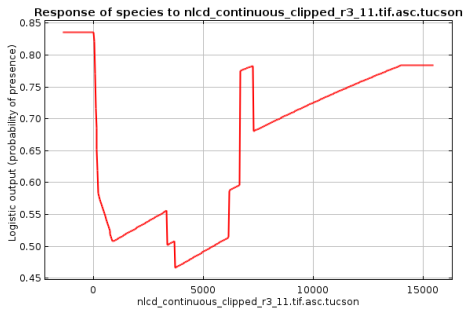
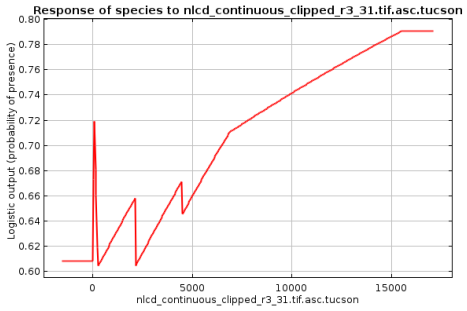
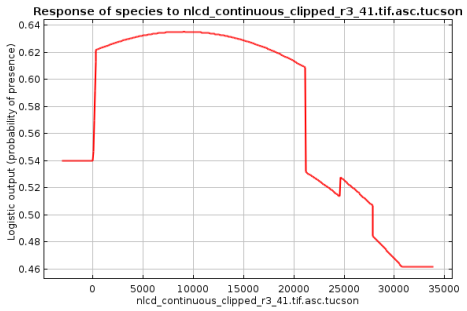
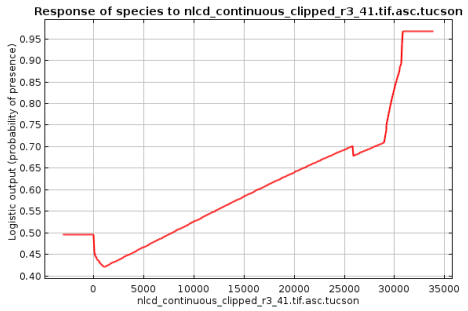
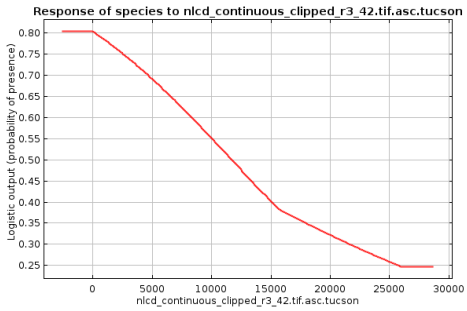
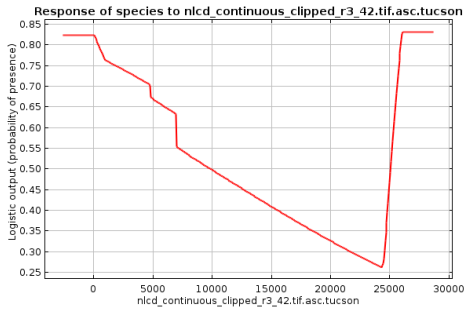
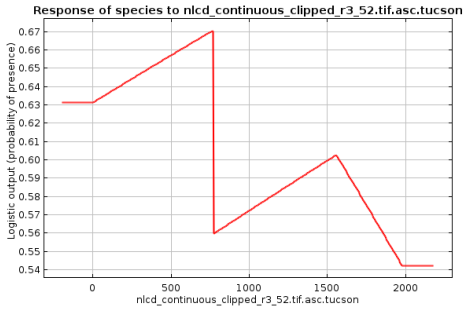
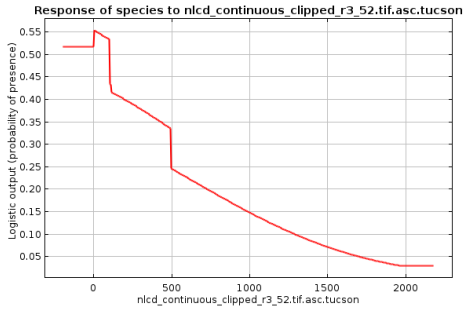
Distance from Cultivated Crops		
Distance from Woody Wetlands		

Figure S2: Marginal Response of Species to Environmental Variables across Tucson

These plots are produced by the Maxent software and depict how each environmental variable affects the maxent prediction of occurrence of the species across the range. These plots display predicted probability of occurrence on the y-axis with all other variables set to their average value.

Variable	Northern Cardinal	Pyrrhuloxia
Climate PC2		

Climate PC3		
Elevation		
% Surface Imperviousness		
Median Household Income		
Canopy		

Distance from Open Water		
Distance from Barren Land		
Distance from Deciduous Forest		
Distance from Evergreen Forest		
Distance from Shrub/Scrub		

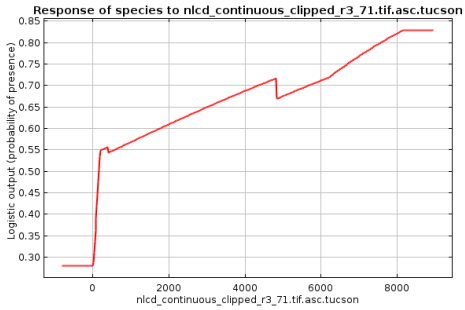
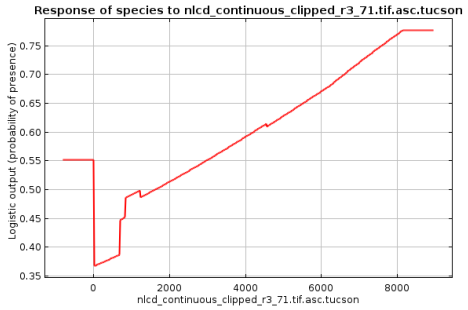
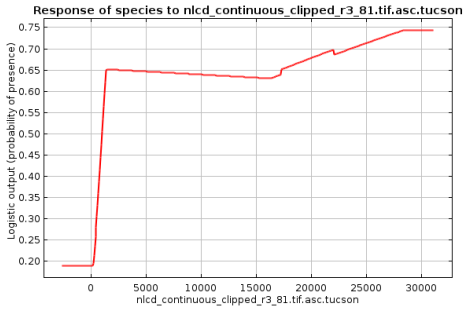
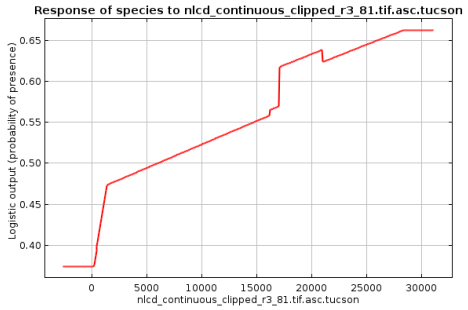
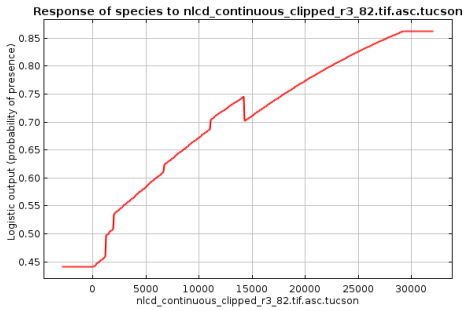
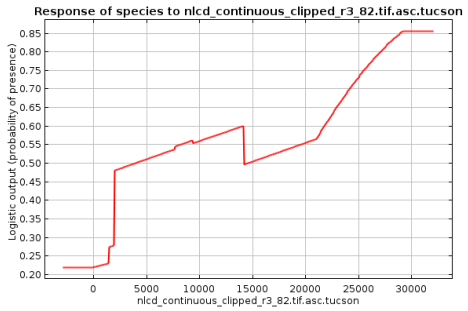
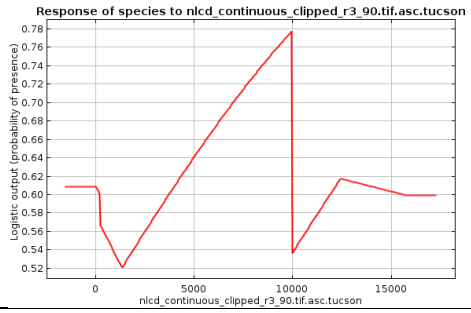
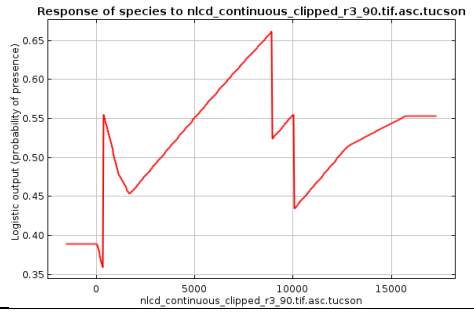
Distance from Grassland/ Herbaceous		
Distance from Pasture/Hay		
Distance from Cultivated Crops		
Distance from Woody Wetlands		

Figure S3: MaxENT Model of Northern Cardinals Across Phoenix.

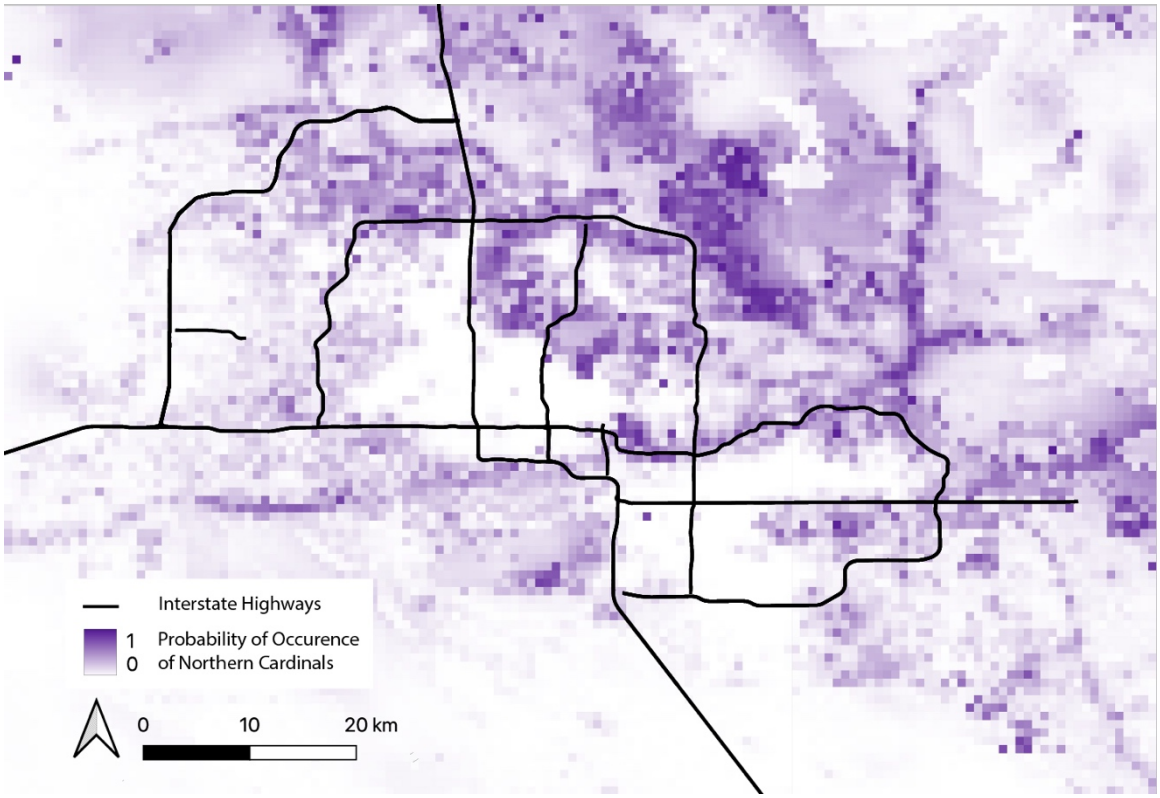
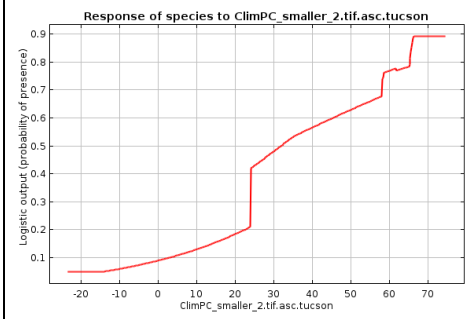
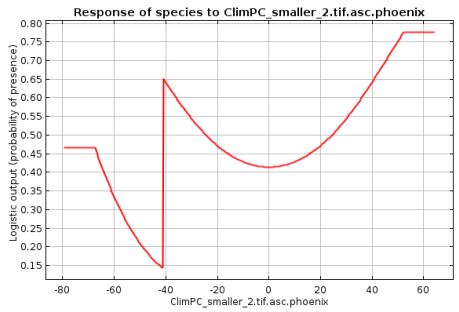
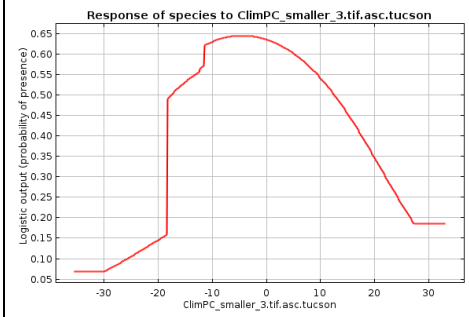
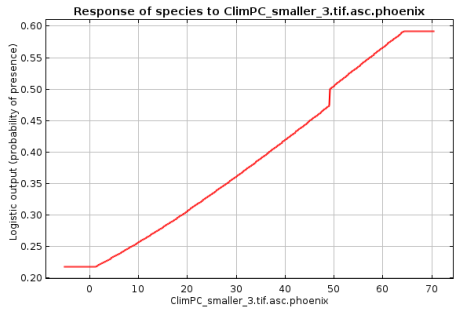
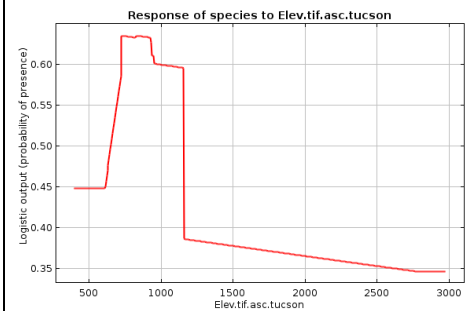
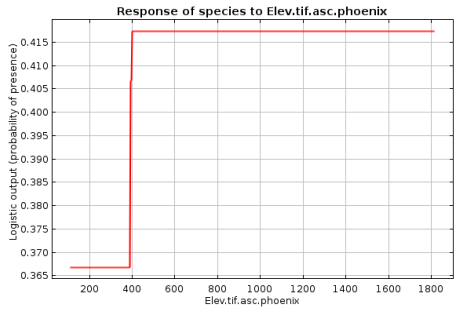
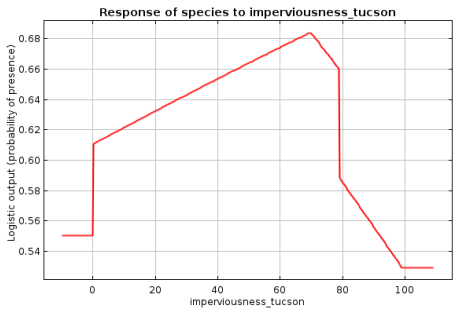
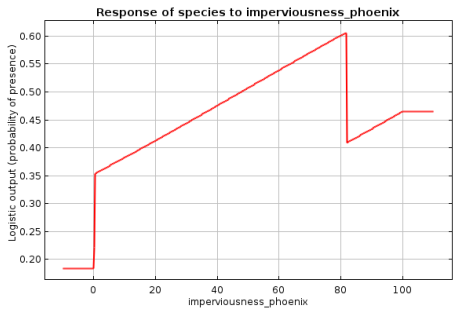
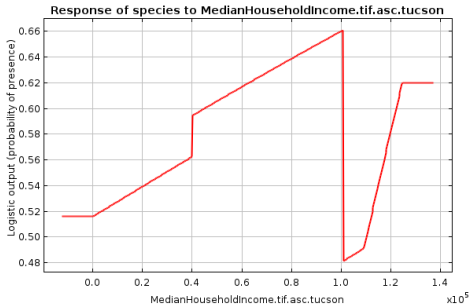
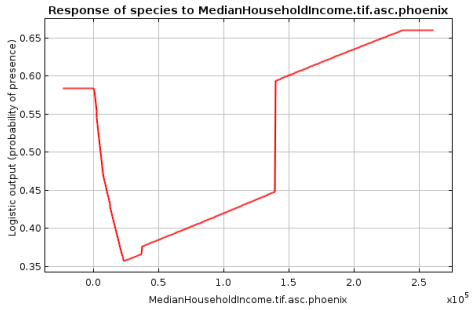
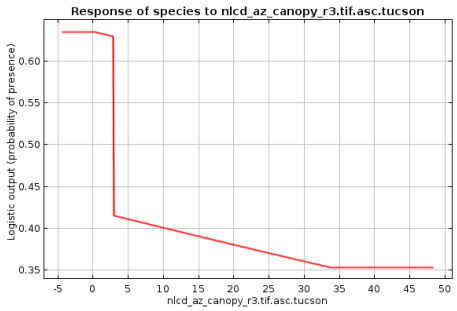
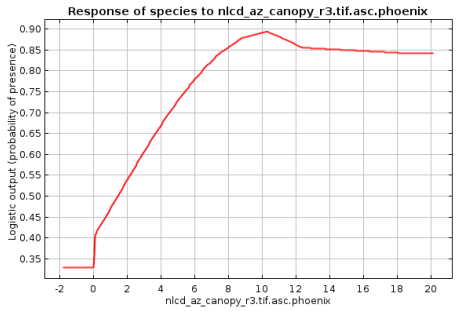
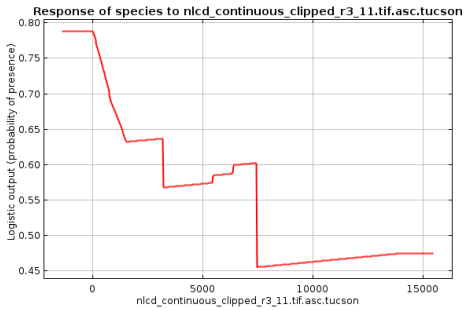
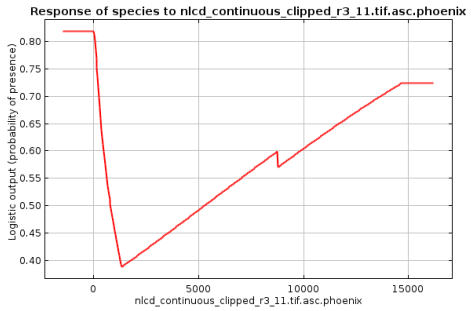
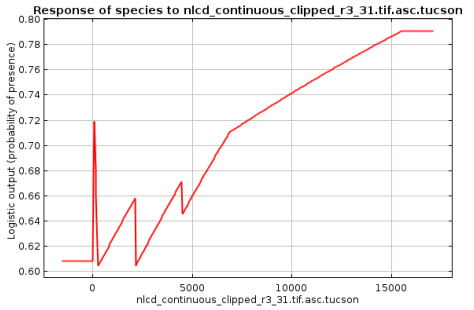
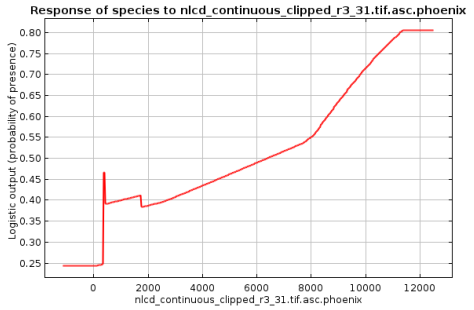
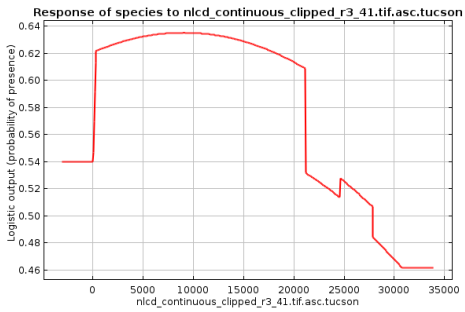
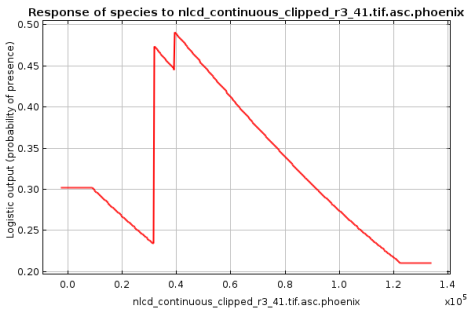
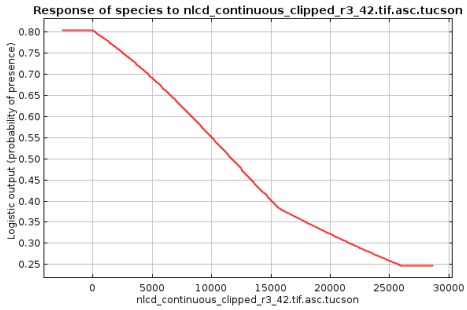
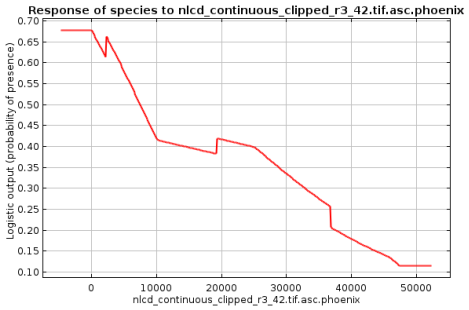
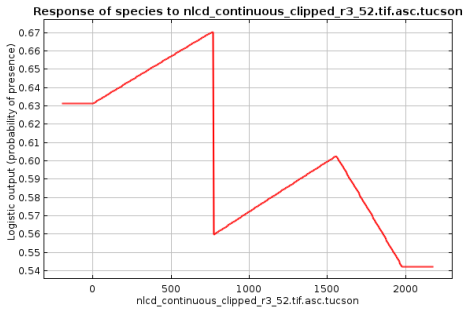
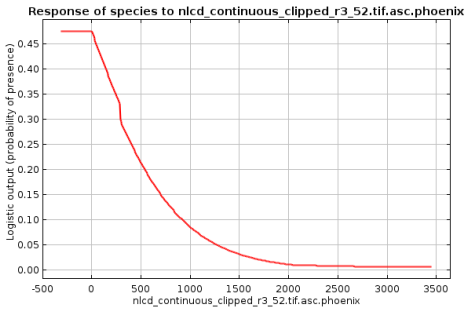
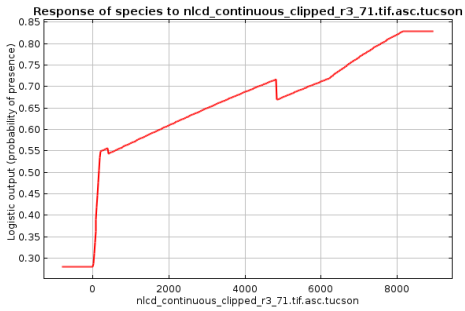
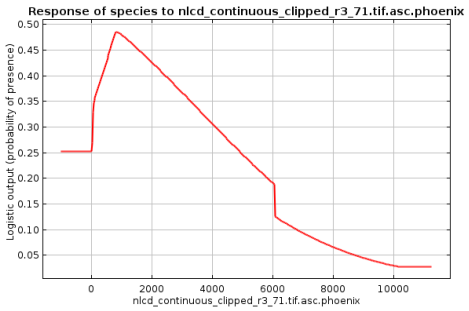
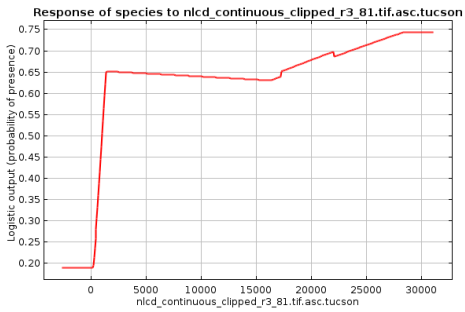
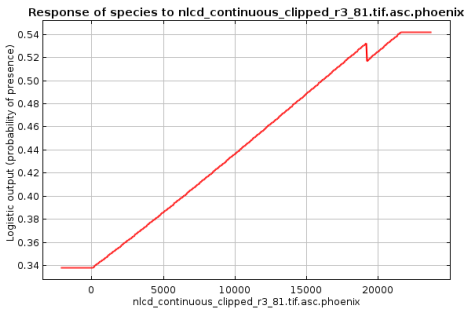


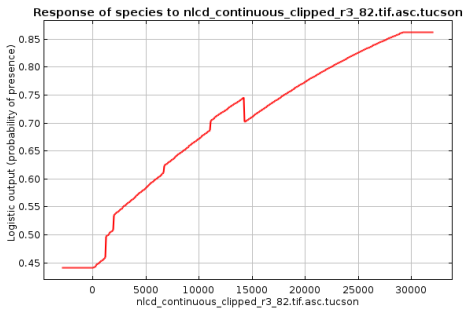
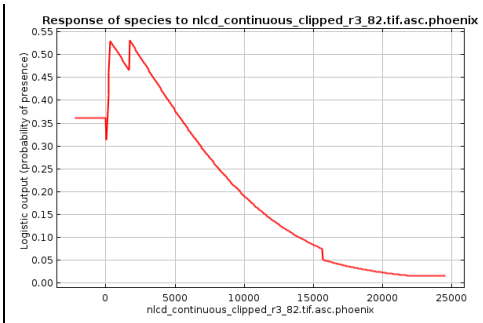
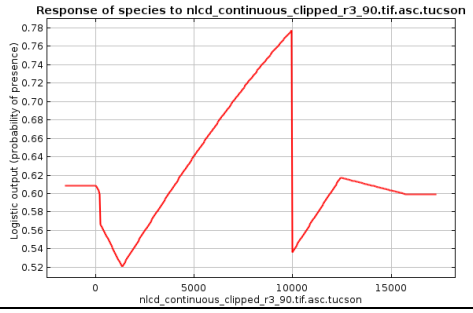
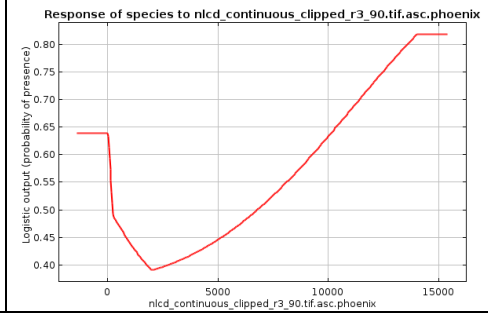
Figure S4: Marginal Response of Northern Cardinals to Environmental Variables across Tucson vs. Phoenix

These plots are produced by the Maxent software and depict how each environmental variable affects the maxent prediction of occurrence of the species across the range. These plots display predicted probability of occurrence on the y-axis with all other variables set to their average value. Tucson plots are identical to those presented in Figure S2, but are represented here for ease of visual comparison.

Variable	Northern Cardinals: Tucson	Northern Cardinals: Phoenix
Climate PC2		
Climate PC3		
Elevation		

% Surface Imperviousness		
Median Household Income		
Canopy		
Distance from Open Water		
Distance from Barren Land		

Distance from Deciduous Forest		
Distance from Evergreen Forest		
Distance from Shrub/Scrub		
Distance from Grassland/ Herbaceous		
Distance from Pasture/Hay		

Distance from Cultivated Crops		
Distance from Woody Wetlands		

Tables

Table S1: Bioclimatic variables legend.

Variable name and associated definition from the WorldClim database (Fick and Hijmans 2017).

Variable	Meaning
Clim1	Annual Mean Temperature
Clim2	Mean Diurnal Range (Mean of monthly (max temp - min temp))
Clim3	Isothermality (BIO2/BIO7) (×100)
Clim4	Temperature Seasonality (standard deviation ×100)
Clim5	Max Temperature of Warmest Month
Clim6	Min Temperature of Coldest Month
Clim7	Temperature Annual Range (BIO5-BIO6)
Clim8	Mean Temperature of Wettest Quarter
Clim9	Mean Temperature of Driest Quarter
Clim10	Mean Temperature of Warmest Quarter
Clim11	Mean Temperature of Coldest Quarter
Clim12	Annual Precipitation
Clim13	Precipitation of Wettest Month
Clim14	Precipitation of Driest Month
Clim15	Precipitation Seasonality (Coefficient of Variation)
Clim16	Precipitation of Wettest Quarter
Clim17	Precipitation of Driest Quarter
Clim18	Precipitation of Warmest Quarter
Clim19	Precipitation of Coldest Quarter

Table S2: NLCD variables legend.

Variables in the NLCD Land Cover classification file. Variables with an asterisk are only relevant to Alaska. Bolded rows were included in this analysis.

Variable	**Meaning**
NLCD 11	**Open Water**
NLCD 12	Perennial Ice/Snow
NLCD 21	Developed, Open Space
NLCD 22	Developed, Low Intensity
NLCD 23	Developed, Medium Intensity
NLCD 24	Developed, High Intensity
NLCD 31	**Barren Land (Rock/Sand/Clay)**
NLCD 41	**Deciduous Forest**
NLCD 42	**Evergreen Forest**
NLCD 43	**Mixed Forest**
NLCD 51	Dwarf Scrub*
NLCD 52	**Shrub/Scrub**
NLCD 71	**Grassland/Herbaceous**
NLCD 72	Sedge/Herbaceous*
NLCD 73	Lichens*
NLCD 74	Moss*
NLCD 81	**Pasture/Hay**
NLCD 82	**Cultivated Crops**
NLCD 90	**Woody Wetlands**
NLCD 95	**Emergent Herbaceous Wetlands**

Table S3: Arizona Correlation Table of Climate Variables.

Pearson's correlation coefficients for all climate variables across the state of Arizona.

	Clim 1	Clim 10	Clim 11	Clim 12	Clim 13	Clim 14	Clim 15	Clim 16	Clim 17	Clim 18	Clim 19	Clim 2	Clim 3	Clim 4	Clim 5	Clim 6	Clim 7	Clim 8	Clim 9
Clim 1	1.00	0.98	0.98	-0.53	-0.39	-0.88	0.35	-0.43	-0.86	-0.47	-0.33	-0.04	0.09	0.03	0.96	0.97	-0.17	0.56	0.99
Clim 10		1.00	0.93	-0.63	-0.53	-0.92	0.23	-0.56	-0.89	-0.59	-0.43	-0.06	-0.05	0.20	0.99	0.92	-0.03	0.53	0.99
Clim 11			1.00	-0.39	-0.24	-0.81	0.45	-0.28	-0.79	-0.32	-0.20	-0.02	0.22	-0.16	0.89	0.98	-0.32	0.56	0.96
Clim 12				1.00	0.90	0.76	0.12	0.92	0.81	0.92	0.94	0.00	0.41	-0.68	-0.67	-0.38	-0.50	-0.34	-0.59
Clim 13					1.00	0.61	0.49	0.99	0.59	0.98	0.75	0.13	0.60	-0.81	-0.58	-0.26	-0.56	-0.16	-0.45
Clim 14						1.00	-0.23	0.65	0.94	0.66	0.61	0.03	0.12	-0.31	-0.91	-0.80	-0.10	-0.55	-0.91
Clim 15							1.00	0.46	-0.34	0.42	-0.02	0.32	0.70	-0.62	0.17	0.37	-0.42	0.36	0.34
Clim 16								1.00	0.63	0.98	0.77	0.13	0.58	-0.78	-0.62	-0.30	-0.54	-0.21	-0.48
Clim 17									1.00	0.66	0.72	-0.04	0.05	-0.29	-0.88	-0.75	-0.12	-0.60	-0.89
Clim 18										1.00	0.77	0.11	0.56	-0.77	-0.64	-0.33	-0.53	-0.20	-0.52
Clim 19											1.00	-0.12	0.30	-0.61	-0.47	-0.17	-0.54	-0.32	-0.41
Clim 2												1.00	0.71	-0.12	0.06	-0.19	0.48	0.16	-0.02
Clim 3													1.00	-0.76	-0.04	0.10	-0.28	0.23	0.04
Clim 4														1.00	0.28	-0.14	0.79	-0.07	0.11
Clim 5															1.00	0.87	0.11	0.51	0.97
Clim 6																1.00	-0.40	0.51	0.94
Clim 7																	1.00	-0.07	-0.09
Clim 8																		1.00	0.56
Clim 9																			1.00

Table S4: Tucson Correlation Table of Climate Variables.

Pearson's correlation coefficients for all climate variables across the city of Tucson.

	Clim 1	Clim 10	Clim 11	Clim 12	Clim 13	Clim 14	Clim 15	Clim 16	Clim 17	Clim 18	Clim 19	Clim 2	Clim 3	Clim 4	Clim 5	Clim 6	Clim 7	Clim 8	Clim 9
Clim 1	1.00	1.00	1.00	-0.98	-0.98	-0.97	0.32	-0.97	-0.98	-0.97	-0.95	0.81	0.68	0.94	0.99	0.94	0.86	1.00	1.00
Clim 10		1.00	0.99	-0.98	-0.98	-0.97	0.33	-0.98	-0.99	-0.98	-0.96	0.82	0.69	0.95	0.99	0.93	0.88	1.00	1.00
Clim 11			1.00	-0.97	-0.96	-0.96	0.33	-0.96	-0.97	-0.95	-0.95	0.79	0.68	0.91	0.98	0.95	0.83	0.99	0.99
Clim 12				1.00	0.98	0.98	-0.40	0.98	1.00	0.97	0.99	-0.87	-0.73	-0.96	-0.99	-0.86	-0.92	-0.98	-0.98
Clim 13					1.00	0.95	-0.22	1.00	0.98	1.00	0.94	-0.78	-0.63	-0.97	-0.96	-0.90	-0.86	-0.98	-0.98
Clim 14						1.00	-0.49	0.94	0.99	0.93	0.98	-0.88	-0.77	-0.93	-0.98	-0.85	-0.92	-0.97	-0.98
Clim 15							1.00	-0.21	-0.40	-0.18	-0.54	0.65	0.72	0.26	0.42	0.14	0.56	0.31	0.34
Clim 16								1.00	0.98	1.00	0.94	-0.79	-0.63	-0.97	-0.96	-0.89	-0.86	-0.98	-0.98
Clim 17									1.00	0.97	0.98	-0.86	-0.73	-0.96	-0.99	-0.87	-0.91	-0.99	-0.99
Clim 18										1.00	0.92	-0.77	-0.60	-0.97	-0.96	-0.89	-0.85	-0.98	-0.97
Clim 19											1.00	-0.90	-0.79	-0.92	-0.98	-0.82	-0.94	-0.96	-0.96
Clim 2												1.00	0.95	0.83	0.89	0.57	0.98	0.82	0.82
Clim 3													1.00	0.66	0.77	0.46	0.88	0.69	0.69
Clim 4														1.00	0.96	0.80	0.92	0.95	0.95
Clim 5															1.00	0.87	0.93	0.99	0.99
Clim 6																1.00	0.63	0.93	0.93
Clim 7																	1.00	0.87	0.87
Clim 8																		1.00	1.00
Clim 9																			1.00

Table S5: Phoenix Correlation Table of Climate Variables.

Pearson's correlation coefficients for all climate variables across the city of Phoenix.

	Clim 1	Clim 10	Clim 11	Clim 12	Clim 13	Clim 14	Clim 15	Clim 16	Clim 17	Clim 18	Clim 19	Clim 2	Clim 3	Clim 4	Clim 5	Clim 6	Clim 7	Clim 8	Clim 9
Clim 1	1.00	0.97	0.94	-0.89	-0.92	-0.85	0.50	-0.91	-0.87	-0.87	-0.87	0.31	-0.03	0.62	0.87	0.57	0.46	0.52	0.98
Clim 10		1.00	0.84	-0.91	-0.92	-0.84	0.62	-0.92	-0.91	-0.88	-0.90	0.40	-0.05	0.78	0.93	0.41	0.61	0.59	0.99
Clim 11			1.00	-0.77	-0.84	-0.74	0.33	-0.80	-0.75	-0.80	-0.74	0.13	0.00	0.32	0.69	0.75	0.20	0.33	0.88
Clim 12				1.00	0.98	0.91	-0.70	0.99	0.99	0.91	0.99	-0.36	0.06	-0.69	-0.81	-0.32	-0.55	-0.72	-0.93
Clim 13					1.00	0.89	-0.58	0.99	0.96	0.93	0.97	-0.36	0.01	-0.62	-0.81	-0.38	-0.51	-0.63	-0.95
Clim 14						1.00	-0.61	0.93	0.92	0.78	0.92	-0.36	0.01	-0.61	-0.76	-0.32	-0.51	-0.71	-0.85
Clim 15							1.00	-0.65	-0.76	-0.60	-0.69	0.28	-0.19	0.67	0.59	0.04	0.52	0.57	0.61
Clim 16								1.00	0.98	0.90	0.99	-0.36	0.04	-0.67	-0.81	-0.34	-0.54	-0.71	-0.94
Clim 17									1.00	0.89	0.99	-0.34	0.11	-0.71	-0.80	-0.31	-0.55	-0.73	-0.92
Clim 18										1.00	0.88	-0.35	-0.01	-0.59	-0.79	-0.38	-0.49	-0.51	-0.91
Clim 19											1.00	-0.35	0.08	-0.69	-0.79	-0.29	-0.55	-0.77	-0.91
Clim 2												1.00	0.77	0.55	0.68	-0.50	0.91	0.21	0.35
Clim 3													1.00	-0.09	0.22	-0.40	0.44	-0.22	-0.06
Clim 4														1.00	0.83	-0.14	0.84	0.64	0.71
Clim 5															1.00	0.15	0.82	0.51	0.90
Clim 6																1.00	-0.44	0.03	0.46
Clim 7																	1.00	0.45	0.54
Clim 8																		1.00	0.57
Clim 9																			1.00

Table S6: Correlation coefficients of environmental variables across the state of Arizona.

Pearson's correlation coefficients for each pair of environmental files across the state of Arizona.

	canopy	ClimPC2	ClimPC3	Elev	imperviousness	MedianHouseholdIncome	NLCD11	NLCD31	NLCD41	NLCD42	NLCD52	NLCD71	NLCD81	NLCD82	NLCD91
canopy	1	-0.13	0 < 0.01	0.46	-0.04	0.08	-0.10	0.25	-0.19	-0.22	0.32	0.03	0.08	0.13	-0.04
ClimPC2		1	0 < 0.01	-0.45	0.06	-0.04	0.14	> -0.01	0.05	0.39	0.05	-0.03	-0.13	-0.34	0.17
ClimPC3			1	-0.37	0.09	0.17	0.19	-0.02	0.06	0.24	0.10	0.28	0.03	-0.35	0.14
Elev				1	-0.12	0.06	-0.19	0.38	-0.21	-0.67	0.06	-0.12	0.27	0.55	-0.04
Imperviousness					1	0.12	-0.10	-0.05	0.06	0.06	0.14	0.07	-0.09	-0.09	-0.06
MedianHouseholdIncome						1	-0.24	0.11	-0.18	-0.22	0.03	0.04	-0.07	-0.01	-0.13
NLCD11							1	-0.11	0.14	0.36	-0.07	0.18	0.21	0.06	0.56
NLCD31								1	-0.17	-0.37	0.04	0.05	0.06	-0.02	0.01
NLCD41									1	0.42	-0.07	-0.12	0.21	0.29	0 > -0.01
NLCD42										1	0.01	-0.08	-0.07	-0.27	0.16
NLCD52											1	0.07	-0.04	-0.02	0 > -0.01
NLCD71												1	0 > -0.01	-0.10	0.09
NLCD81													1	0.49	0.33
NLCD82														1	0.09
NLCD90															1

Table S7: Correlation coefficients of environmental variables across the Tucson metropolitan area.

Pearson's correlation coefficients for each pair of environmental files across the Tucson region.

	canopy	ClimPC2	ClimPC3	Elev	imperviousness	MedianHouseholdIncome	NLCD11	NLCD31	NLCD41	NLCD42	NLCD52	NLCD71	NLCD81	NLCD82	NLCD91
canopy	1	-0.60	-0.81	0.32	-0.08	-0.42	-0.60	-0.74	0.01	0.58	0.14	-0.12	-0.30	-0.70	-0.14
ClimPC2		1	0.25	-0.07	0.26	0.03	0.23	0.24	0.26	-0.45	-0.04	0.37	0.32	0.27	0.25
ClimPC3			1	-0.28	0.04	0.58	0.51	0.79	-0.28	-0.53	-0.12	-0.03	0.03	0.67	-0.04
Elev				1	-0.21	-0.12	-0.23	-0.31	-0.15	0.13	0.54	0.12	-0.01	-0.27	-0.10
Imperviousness					1	0<0.01	-0.08	0.01	0.15	-0.38	-0.28	0.26	0.12	0>-0.01	-0.06
MedianHouseholdIncome						1	0.30	0.49	-0.15	-0.23	-0.02	-0.02	0<0.01	0.31	-0.15
NLCD11							1	0.69	0.04	-0.23	-0.05	0.03	0.20	0.57	0.33
NLCD31								1	0<0.01	-0.34	-0.09	-0.01	0.06	0.56	0.05
NLCD41									1	0.39	-0.11	0.17	0.34	-0.22	0.35
NLCD42										1	0.08	-0.28	-0.02	-0.38	0.12
NLCD52											1	0.12	-0.04	-0.09	-0.01
NLCD71												1	0.18	-0.10	0.15
NLCD81													1	0.36	0.38
NLCD82														1	0.29
NLCD90															1

Table S8: Correlation coefficients of environmental variables across the Phoenix metropolitan area.

Pearson's correlation coefficients for each pair of environmental files across the Phoenix region.

	canopy	ClimPC2	ClimPC3	Elev	imperviousness	MedianHouseholdIncome	NLCD11	NLCD31	NLCD41	NLCD42	NLCD52	NLCD71	NLCD81	NLCD82	NLCD91
canopy	1	-0.32	-0.74	0.47	-0.34	-0.01	-0.30	-0.12	0.62	0.63	0.43	0.06	-0.35	-0.75	-0.11
ClimPC2		1	0.63	0.11	0.56	0.05	-0.10	0.47	-0.84	-0.25	0.05	0.39	0.39	0.43	0.12
ClimPC3			1	-0.27	0.49	0.03	0.34	0.38	-0.68	-0.54	-0.27	0.26	0.47	0.77	0.32
Elev				1	-0.03	-0.01	-0.28	0.23	0.07	0.34	0.54	0.33	-0.03	-0.32	-0.05
Imperviousness					1	0.03	-0.08	0.28	-0.58	-0.31	-0.10	0.21	0.35	0.41	0.10
MedianHouseholdIncome						1	-0.05	0.02	-0.06	0<0.01	0.05	0<0.01	-0.04	-0.01	-0.03
NLCD11							1	0.16	0.11	-0.27	-0.18	0.08	0.34	0.39	0.48
NLCD31								1	-0.31	-0.14	0.28	0.60	0.36	0.36	0.22
NLCD41									1	0.30	0.11	-0.17	-0.37	-0.54	-0.02
NLCD42										1	0.37	-0.04	-0.29	-0.59	0.02
NLCD52											1	0.38	-0.09	-0.27	-0.03
NLCD71												1	0.19	0.16	0.22
NLCD81													1	0.51	0.28
NLCD82														1	0.19
NLCD90															1

Table S9: Hypothesis testing for differences in range and environmental niche between northern cardinals and pyrrhuloxia.

Results of identity tests of the range and of the environmental niche of the two species. These test the null hypothesis that either the range or the niche of northern cardinals and pyrrhuloxia are similar. A p-value of < 0.05 is considered significant and is represented in bold.

	Tucson		***Arizona***	
Test statistic	**Value**	**p-value**	**Value**	**p-value**
D	**0.74**	**0.02**	**0.57**	**0.01**
I	**0.94**	**0.03**	**0.85**	**0.01**
Rank Correlation	**0.81**	**0.05**	**0.69**	**0.01**
D env	0.43	0.07	0.47	0.07
I env	0.74	0.08	0.74	**0.05**
Rank Correlation env	0.14	0.20	0.57	0.22

Table S10: Comparison of Environmental Variable Means for Northern Cardinals Across Tucson vs. Northern Cardinals Across Phoenix.

Means, standard deviations, and results of the t-test for the difference in means between the species are shown for each variable. Elevation is in meters. All units for NLDC files are in meters. Climate temperatures are in Celsius and precipitation values are in millimeters. Rows with significant p-values are bolded.

Environmental variable	Northern cardinal Tucson mean	Northern cardinal Tucson SD	Northern Cardinal Phoenix mean	Northern Cardinal Phoenix SD	T	DF	P
Canopy	0.686	3.471	0.327	1.560	2.047	803	0.041
Elevation	**826.538**	**84.225**	**513.571**	**149.415**	**29.817**	**282**	**< 0.001**
Imperviousness	22.774	25.811	20.764	26.114	0.991	403	0.322
Median Household Income	**$79665.67**	**$21707.97**	**$96791.22**	**$41259.33**	**-5.950**	**275**	**< 0.001**
NLCD 11: Open Water	**2585.761**	**1664.41**	**2140.934**	**2211.473**	**2.750**	**328**	**0.006**
NLCD 31: Barren Land	**1197.144**	**997.487**	**3302.177**	**2335.712**	**-13.133**	**258**	**< 0.001**
NLCD 41: Deciduous Forest	**8782.485**	**7450.849**	**56988.729**	**16892.429**	**-41.496**	**260**	**< 0.001**
NLCD 42: Evergreen Forest	**6572.557**	**5870.968**	**19709.751**	**10138.277**	**-18.391**	**285**	**< 0.001**
NLCD 52: Shrub/Scrub	87.329	204.215	116.990	207.530	-1.842	402	0.066
NLCD 71: Grassland/Herbaceous	**2731.934**	**1590.360**	**2331.430**	**1812.115**	**2.930**	**365**	**0.004**
NLCD 81: Pasture/Hay	**12400.445**	**5715.352**	**7607.657**	**5174.498**	**11.543**	**447**	**< 0.001**
NLCD 82: Cultivated Crops	**7445.950**	**3992.819**	**4440.339**	**4391.448**	**9.000**	**376**	**< 0.001**
NLCD 90: Woody Wetlands	**4358.030**	**2616.696**	**3290.193**	**2927.805**	**4.815**	**370**	**< 0.001**
Clim 1: Annual Mean Temperature	**20.139**	**0.552**	**21.312**	**0.820**	**-19.922**	**307**	**< 0.001**

Clim 2: Mean Diurnal Range	**15.352**	**0.594**	**16.448**	**0.553**	**-24.918**	**435**	**< 0.001**
Clim 3: Isothermality	**45.605**	**0.832**	**45.168**	**0.799**	**6.942**	**423**	**< 0.001**
Clim 4: Temperature Seasonality	**733.621**	**12.470**	**783.613**	**16.410**	**-41.581**	**330**	**< 0.001**
Clim 5: Max Temp of Warmest Month	**37.302**	**0.796**	**40.456**	**0.857**	**-48.145**	**382**	**< 0.001**
Clim 6: Min Temp of Coldest Month	**3.650**	**0.415**	**4.047**	**0.714**	**-7.901**	**286**	**< 0.001**
Clim 7: Temperature Annual Range	**33.652**	**0.816**	**36.409**	**0.843**	**-42.314**	**396**	**< 0.001**
Clim 8: Mean Temp of Wettest Quarter	**28.680**	**0.658**	**16.630**	**7.056**	**25.687**	**227**	**< 0.001**
Clim 9: Mean Temp of Driest Quarter	**23.434**	**0.649**	**24.310**	**1.069**	**-11.555**	**291**	**< 0.001**
Clim 10: Mean Temp of Warmest Quarter	**29.222**	**0.676**	**31.044**	**0.933**	**-26.866**	**320**	**< 0.001**
Clim 11: Mean Temp of Coldest Quarter	**11.496**	**0.456**	**12.145**	**0.698**	**-12.985**	**302**	**< 0.001**
Clim 12: Annual Precipitation	**338.882**	**31.530**	**303.018**	**69.440**	**7.495**	**262**	**< 0.001**
Clim 13: Precipitation of Wettest Month	**60.741**	**4.707**	**39.726**	**7.594**	**38.960**	**294**	**< 0.001**
Clim 14: Precipitation of Driest Month	**5.586**	**0.644**	**3.580**	**1.261**	**22.874**	**272**	**< 0.001**
Clim 15: Precipitation Seasonality	**60.663**	**3.145**	**46.941**	**1.503**	**84.408**	**784**	**< 0.001**
Clim 16: Precipitation of Wettest Quarter	**154.386**	**12.749**	**102.204**	**22.151**	**33.457**	**285**	**< 0.001**
Clim 17: Precipitation of Driest Quarter	**21.604**	**2.212**	**20.469**	**5.957**	**2.796**	**250**	**0.006**
Clim 18: Precipitation of Warmest Quarter	**125.569**	**10.693**	**72.949**	**19.720**	**38.138**	**278**	**< 0.001**
Clim 19: Precipitation of Coldest Quarter	**85.651**	**10.417**	**99.531**	**23.766**	**-8.496**	**260**	**< 0.001**

APPENDIX C

CHAPTER 3 SUPPLEMENTARY TABLES AND FIGURES

Figures

Figure S1. *Specimen sampling by species, sex, urban category, and year.*

We sampled male northern cardinals from urban (N=15), urban outskirts (N=8), and rural regions (N=16), female northern cardinals from urban and urban outskirt regions (N=4) and from rural regions (N=5), male pyrrhuloxia from urban (N=13), urban outskirts (N=11), and rural regions (N=10), and female pyrrhuloxia from urban and urban outskirt regions (N=13), and from rural regions (N=9). These samples span 137 years, from 1885 to 2022, with all subgroups of sex and urban category containing sampling across many decades.

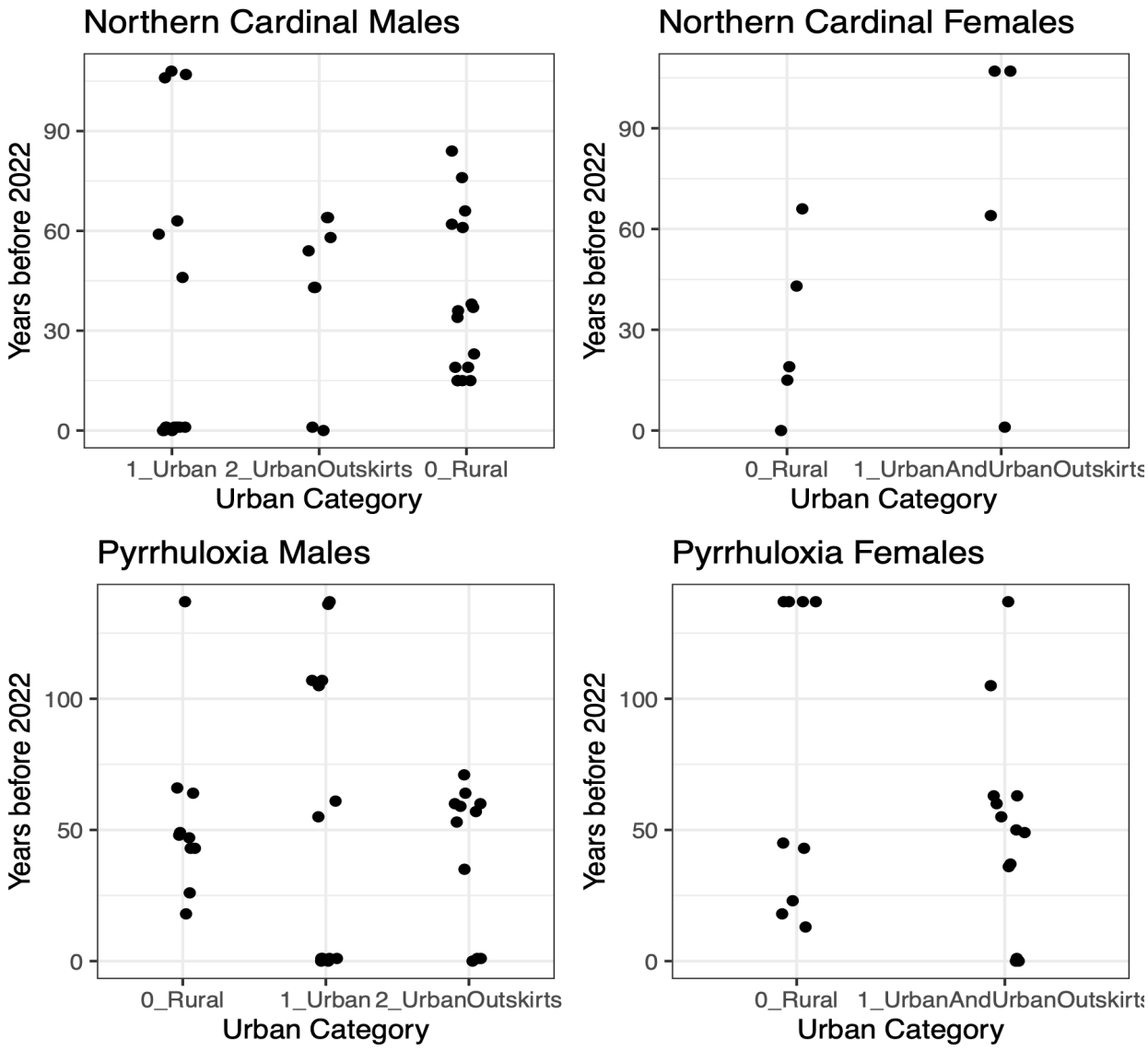
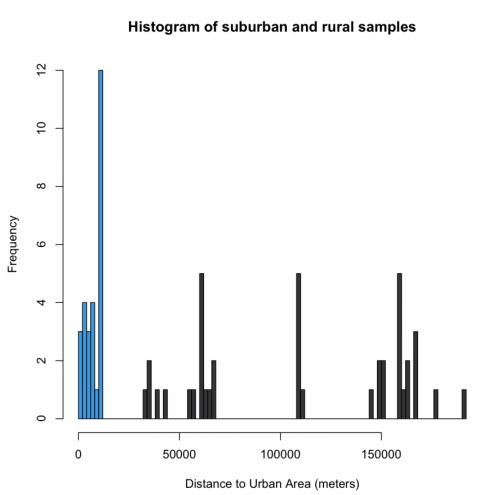

Northern Cardinal Males
Years before 2022
90
60
30
0
1_Urban
2_UrbanOutskirts
0_Rural
Urban Category
Northern Cardinal Females
Years before 2022
90
60
30
0
0_Rural
1_UrbanAndUrbanOutskirts
Urban Category
Pyrrhuloxia Males
Years before 2022
100
50
0
0_Rural
1_Urban
2_UrbanOutskirts
Urban Category
Pyrrhuloxia Females
Years before 2022
100
50
0
0_Rural
1_UrbanAndUrbanOutskirts
Urban Category

Figure S2. Sampling locations and urban categories.

Top: Histogram of urban outskirt and rural samples by distance to urban area. Blue samples are urban outskirt samples and black samples are rural samples. Urban samples were excluded from the plot as they are all at a distance of 0 from the urban area.

Bottom: Sample locations by urban category. Purple pins are urban, yellow/green pins are urban outskirts, dark green pins are rural. The left map shows samples around Tucson and the right map shows samples across Arizona.



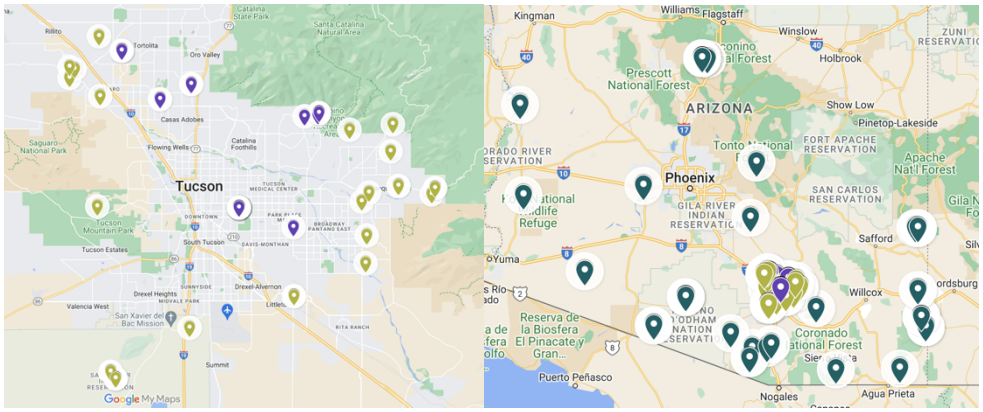
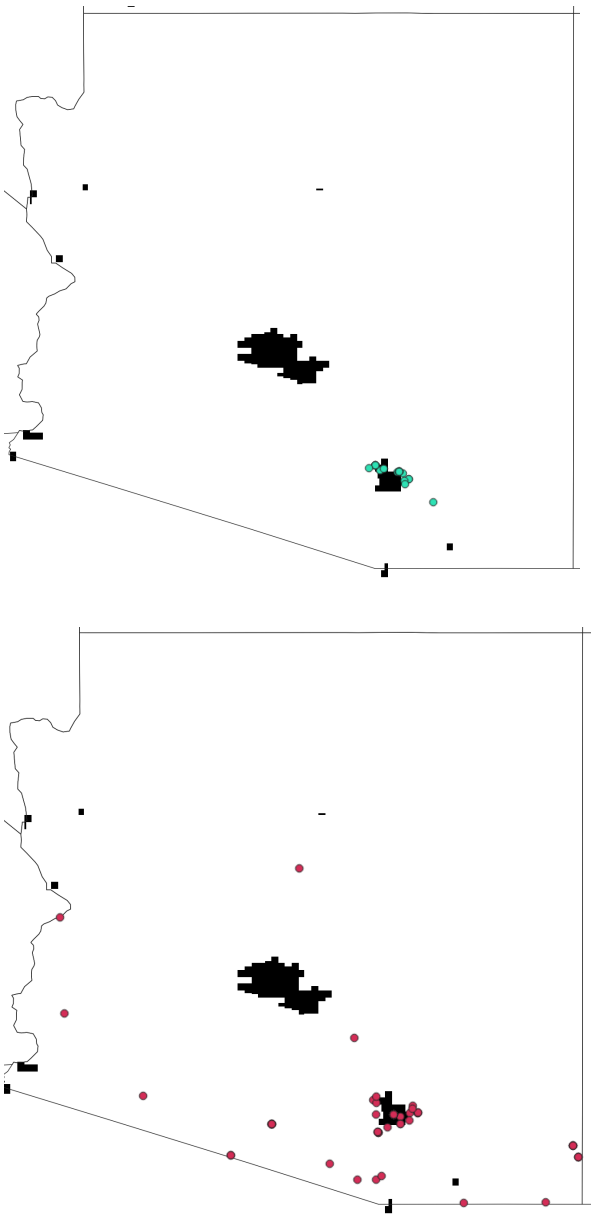


Figure S3. Sample locations by source and model of 2020 urban areas.
In each plot, the black squares represent urban areas and the points represent sampled birds. The top plot shows field samples, the middle plot shows sampling locations of the University of Arizona Museum specimens, and the bottom plot shows sampling locations of the University of Washington Burke Museum specimens.



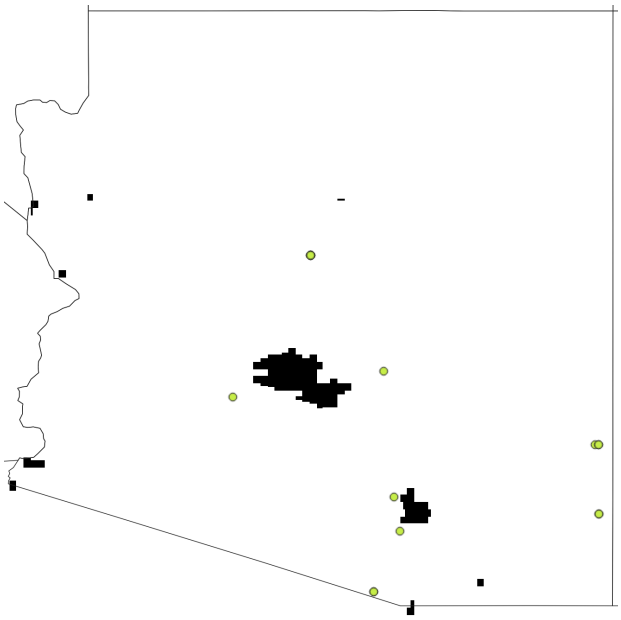
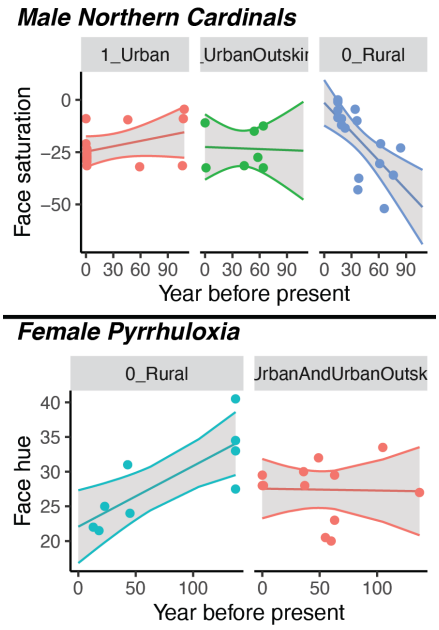


Figure S4: Interaction of year and urbanization on color traits.

The linear regressions with interquartile ranges of each trait size are presented with actual trait measurements of the specimen plotted as points. We only present the two traits for which both urbanization and year or the interaction between the two were significant, which were face saturation in male northern cardinals and face hue for female pyrrhuloxia.



Tables

Table S1: Specimen counts.

For museum specimens, Specimen ID/Accession Numbers associate with museum specimen records in public databases.

Source	Species	Sex	Count	Specimen ID/Accession Number
Field	Northern cardinals	Female	2	NOCA_005, NOCA_009
		Male	11	NOCA_001, NOCA_002, NOCA_003, NOCA_004, NOCA_006, NOCA_007, NOCA_008, NOCA_010, NOCA_011, NOCA_012, NOCA_013
	Pyrrhuloxia	Female	3	PYRR_003, PYRR_009, PYRR_011
		Male	9	PYRR_001, PYRR_002, PYRR_004, PYRR_005, PYRR_006, PYRR_007, PYRR_008, PYRR_010, PYRR_012
UAZ	Northern cardinals	Female	5	UAz-010962, UA z-010966, UAz-013639, UAz-002322, UAz-005162
		Male	19	UAz-010963, UAz-010964, UAz-010965, UAz-012574, UAz-012708, UAz-012709, UAz-012729, UAz-013573, UAz-013861, UAz-014935, UAz-015113, UAz-015691, UAz-001775, UAz-002033, UAz-002891, UAz-003981, UAz-004149, UAz-005161, UAz-009287
	Pyrrhuloxia	Female	17	UAz-010582, UAz-010967, UAz-011316, UAz-012528, UAz-013689, UAz-014709, UAz-015119, UAz-018200, UAz-002034, UAz-002035, UAz-002632, UAz-002633, UAz-002634, UAz-002635, UAz-002636, UAz-004744, UAz-009847
		Male	22	UAz-010968, UAz-010969, UAz-010970, UAz-011409, UAz-012058, UAz-012529, UAz-012733, UAz-013636, UAz-013690, UAz-015334, UAz-001696, UAz-017492, UAz-002627, UAz-002629, UAz-002630, UAz-002892, UAz-004150, UAz-004436, UAz-004743, UAz-005163, UAz-009848, UAz-009908
UWBM	Northern cardinals	Female	2	UWBM Bird 100623, UWBM Bird 77974
		Male	9	UWBM Bird 100619, UWBM Bird 100620, UWBM Bird 100621, UWBM Bird 100622, UWBM Bird 103345, UWBM Bird 48445, UWBM Bird 48493, UWBM Bird 77856, UWBM Bird 77978
	Pyrrhuloxia	Female	2	UWBM Bird 103346, UWBM Bird 77780
		Male	3	UWBM Bird 106398, UWBM Bird 121175, UWBM Bird 77718

Table S2: NOCA M Correlation Analysis r-values

Significant values ($p < 0.05$) are shaded in blue. N values range from 34-38. Many of the length variables were significantly intercorrelated for male northern cardinals, and northern cardinal males had the most intercorrelated traits of the four sex and species subgroups.

	TailLength	Crest	Wing	BillLength	Bill Width	Crest Hue	Crest Saturation	Crest Brightness	Face Hue	Face Saturation	Face Brightness	BreastHue	BreastSaturation	BreastBrightness
TailLength	1.00	0.39	0.36	0.15	0.56	0.13	0.00	0.10	-0.55	-0.05	-0.43	-0.16	0.07	-0.12
Crest		1.00	0.21	-0.03	0.44	-0.21	0.10	0.01	-0.36	-0.20	-0.07	-0.35	-0.04	-0.11
Wing			1.00	-0.23	0.13	-0.19	0.14	0.18	-0.13	-0.16	0.03	-0.39	0.21	0.00
BillLength				1.00	0.08	0.12	0.24	0.45	-0.17	0.52	-0.24	0.17	0.37	-0.05
BillWidth					1.00	0.08	-0.12	-0.03	-0.55	-0.24	-0.20	-0.29	0.00	-0.20
CrestHue						1.00	-0.60	0.13	-0.15	-0.02	-0.19	0.53	-0.28	0.20
CrestSaturation							1.00	-0.05	0.26	0.44	-0.04	-0.23	0.66	-0.32
CrestBrightness								1.00	-0.18	0.19	0.08	-0.09	0.20	0.45
FaceHue									1.00	0.20	0.49	0.02	0.24	0.05
FaceSaturation										1.00	-0.34	0.11	0.58	-0.17
FaceBrightness											1.00	-0.19	-0.11	0.33
BreastHue												1.00	-0.31	0.06
BreastSaturation													1.00	-0.26
BreastBrightness														1.00

Table S3: NOCA F Correlation Analysis r-values

Significant values ($p < 0.05$) are shaded in blue. N values range from 8-9. Fewer length and color variables were intercorrelated for female northern cardinals than for male northern cardinals.

	TailLength	Crest	Wing	BillLength	BillWidth	Crest Hue	Crest Saturation	Crest Brightness	Face Hue	FaceSaturation	Face Brightness	BreastHue	BreastSaturation	BreastBrightness
TailLength	1.00	0.54	-0.03	-0.60	0.85	-0.15	-0.51	0.33	-0.49	-0.65	-0.02	0.36	-0.15	-0.13
Crest		1.00	0.72	-0.51	0.78	-0.02	0.12	-0.64	-0.54	0.02	-0.55	0.33	-0.39	-0.01
Wing			1.00	-0.42	0.33	0.12	0.28	-0.79	-0.36	0.25	-0.46	0.39	-0.53	-0.13
BillLength				1.00	-0.43	0.29	0.50	0.11	0.14	0.62	-0.19	-0.63	0.74	-0.04
BillWidth					1.00	0.04	-0.16	-0.11	-0.81	-0.20	-0.54	0.29	-0.09	-0.28
Crest Hue						1.00	0.14	-0.06	0.02	0.54	-0.14	-0.29	0.50	-0.90
Crest Saturation							1.00	-0.43	-0.31	0.53	-0.57	-0.13	0.05	0.13
Crest Brightness								1.00	0.07	-0.61	0.32	-0.33	0.43	-0.15
Face Hue									1.00	0.16	0.79	-0.18	0.07	0.18
FaceSaturation										1.00	-0.28	-0.25	0.32	-0.23
Face Brightness											1.00	0.29	-0.24	0.17
BreastHue												1.00	-0.89	0.20
BreastSaturation													1.00	-0.44
BreastBrightness														1.00

Table S4: PYRR M Correlation Analysis r-values

Significant values ($p < 0.05$) are shaded in blue. N values range from 26-30. No length variables were correlated in male pyrrhuloxia.

	TailLength	Crest	Wing	BillLength	BillWidth	Crest Hue	Crest Saturation	Crest Brightness	Face Hue	FaceSaturation	Face Brightness	BreastHue	BreastSaturation	BreastBrightness
TailLength	1.00	0.07	0.02	-0.04	0.07	-0.43	-0.13	-0.13	0.02	0.06	-0.21	-0.28	-0.22	-0.24
Crest		1.00	0.12	-0.21	0.11	-0.10	-0.16	-0.23	0.16	-0.40	-0.14	-0.29	-0.24	-0.12
Wing			1.00	-0.17	-0.07	0.07	-0.01	-0.02	0.05	0.06	0.22	-0.17	0.10	0.15
BillLength				1.00	-0.27	0.32	0.03	0.22	0.17	0.24	-0.26	0.06	0.39	0.01
BillWidth					1.00	-0.12	-0.28	-0.32	0.13	-0.32	0.05	-0.26	-0.34	-0.24
Crest Hue						1.00	-0.16	0.26	0.53	-0.22	0.12	0.44	0.17	-0.05
Crest Saturation							1.00	-0.17	-0.50	0.38	-0.17	0.09	0.36	-0.05
Crest Brightness								1.00	0.11	-0.03	0.40	0.10	0.08	0.30
Face Hue									1.00	-0.38	-0.05	0.25	-0.22	-0.06
FaceSaturation										1.00	-0.31	0.07	0.36	0.04
Face Brightness											1.00	-0.06	-0.15	0.23
BreastHue												1.00	-0.04	0.22
BreastSaturation													1.00	-0.25
BreastBrightness														1.00

Table S5: PYRR F Correlation Analysis r-values

Significant values ($p < 0.05$) are shaded in blue. N values range from 19-22. Of the length variables, only crest and tail length were correlated in female pyrrhuloxia. Color traits were highly intercorrelated with each other and only rarely correlated with length variables.

	TailLength	Crest	Wing	BillLength	Bill Width	Crest Hue	Crest Saturation	Crest Brightness	Face Hue	Face Saturation	Face Brightness	BreastHue	BreastSaturation	BreastBrightness
TailLength	1.00	0.56	0.38	0.25	-0.14	-0.14	0.20	-0.54	-0.08	0.03	-0.28	-0.05	0.06	0.04
Crest		1.00	0.26	-0.02	0.14	-0.61	0.25	-0.58	-0.22	0.13	-0.28	-0.12	0.17	-0.04
Wing			1.00	0.03	-0.10	-0.32	-0.05	-0.16	-0.23	0.24	-0.19	-0.08	0.00	0.06
BillLength				1.00	-0.30	0.37	-0.12	0.18	0.02	-0.32	0.15	-0.32	0.11	0.07
BillWidth					1.00	-0.37	0.02	-0.13	-0.01	-0.12	-0.19	-0.08	-0.16	-0.28
CrestHue						1.00	-0.06	0.40	0.15	-0.04	0.30	0.32	-0.34	0.55
CrestSaturation							1.00	-0.60	-0.36	0.51	-0.33	-0.39	0.39	0.22
CrestBrightness								1.00	0.48	-0.45	0.73	0.55	-0.48	0.40
FaceHue									1.00	-0.48	0.60	0.41	-0.41	0.16
FaceSaturation										1.00	-0.59	-0.22	0.18	0.14
FaceBrightness											1.00	0.50	-0.44	0.42
BreastHue												1.00	-0.75	0.63
BreastSaturation													1.00	-0.60
BreastBrightness														1.00

Table S6: Repeatability of photograph measurements (ICC3).
ICC values range from 0.79-1.00.

			H	S	B
Northern cardinals	Male	Crest	0.98	0.91	0.89
		Face	0.90	0.92	0.86
		Breast	0.97	0.97	0.80
	Female	Crest	0.93	0.86	0.98
		Face	0.84	1.00	0.93
		Breast	0.99	0.99	0.98
Pyrrhuloxia	Male	Crest	0.90	0.86	0.83
		Face	0.95	0.89	0.95
		Breast	0.79	0.95	0.80
	Female	Crest	0.88	0.95	0.79
		Face	0.80	0.94	0.83
		Breast	0.96	0.99	0.95
Both	Average		0.91	0.94	0.88
	Total average		0.91		

Table S7: Northern Cardinal Male Coloration Traits ANOVA Table.
Output of each ANOVA model on color traits.

Patch/trait	Variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Crest						
Hue						
	Urban_categorical	2	42.97	21.48	0.60	0.55
	Year_Adj	1	27.73	27.73	0.78	0.39
	Urban_categorical:Year_Adj	2	44.17	22.08	0.62	0.55
	Residuals	30	1070.82	35.69		
Saturation						
	Urban_categorical	2	93.83	46.92	0.64	0.54
	Year_Adj	1	164.04	164.04	2.23	0.15
	Urban_categorical:Year_Adj	2	27.64	13.82	0.19	0.83
	Residuals	30	2204.30	73.48		
Brightness						
	Urban_categorical	2	226.02	113.01	2.32	0.12
	Year_Adj	1	310.84	310.84	6.38	0.02
	Urban_categorical:Year_Adj	2	27.34	13.67	0.28	0.76
	Residuals	30	1461.22	48.71		
Face						
Hue						
	Urban_categorical	2	2049.20	1024.61	7.31	0.00
	Year_Adj	1	2297.50	2297.52	16.38	0.00
	Urban_categorical:Year_Adj	2	317.50	158.74	1.13	0.34
	Residuals	31	4347.50	140.24		
Saturation						
	Urban_categorical	2	106.90	53.44	0.44	0.65
	Year_Adj	1	74.80	74.81	0.62	0.44
	Urban_categorical:Year_Adj	2	1940.60	970.29	8.04	0.00

	Residuals	31	3741.10	120.68		
Brightness						
	Urban_categorical	2	188.59	94.30	1.75	0.19
	Year_Adj	1	722.53	722.53	13.39	0.00
	Urban_categorical:Year_Adj	2	35.92	17.96	0.33	0.72
	Residuals	31	1672.65	53.96		
Breast						
Hue						
	Urban_categorical	2	11.23	5.62	0.47	0.63
	Year_Adj	1	19.58	19.58	1.65	0.21
	Urban_categorical:Year_Adj	2	28.86	14.43	1.22	0.31
	Residuals	31	367.13	11.84		
Saturation						
	Urban_categorical	2	44.30	22.15	0.29	0.75
	Year_Adj	1	42.49	42.49	0.55	0.46
	Urban_categorical:Year_Adj	2	188.20	94.10	1.23	0.31
	Residuals	31	2377.81	76.70		
Brightness						
	Urban_categorical	2	291.56	145.78	4.06	0.03
	Year_Adj	1	0.00	0.00	0.00	1.00
	Urban_categorical:Year_Adj	2	17.73	8.86	0.25	0.78
	Residuals	31	1114.24	35.94		

Table S8: Northern Cardinal Female Coloration Traits ANOVA Table

Patch/trait	Variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Crest						
Hue						
	Urban_categorical	1	50.00	50.00	1.27	0.30
	Residuals	6	236.38	39.40		
Saturation						
	Urban_categorical	1	0.03	0.03	0.00	0.99
	Residuals	6	683.94	113.99		
Brightness						
	Urban_categorical	1	72.00	72.00	1.88	0.22
	Residuals	6	229.38	38.23		
Face						
Hue						
	Urban_categorical	1	0.50	0.50	0.01	0.91
	Residuals	6	220.00	36.67		
Saturation						
	Urban_categorical	1	4.50	4.50	0.01	0.91
	Residuals	6	1994.00	332.33		
Brightness						
	Urban_categorical	1	66.13	66.13	0.73	0.42
	Residuals	6	540.87	90.15		
Breast						
Hue						
	Urban_categorical	1	105.12	105.13	3.54	0.11
	Residuals	6	178.38	29.73		
Saturation						
	Urban_categorical	1	253.12	253.13	2.92	0.14
	Residuals	6	519.75	86.63		

Brightness						
	Urban_categorical	1	7.03	7.03	0.16	0.71
	Residuals	6	269.94	44.99		

Table S9: Pyrrhuloxia Male Coloration Traits ANOVA Table

Patch/trait	Variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Crest						
Hue						
	Urban_categorical	2	48.13	24.07	0.49	0.62
	Year_Adj	1	31.04	31.04	0.64	0.43
	Urban_categorical:Year_Adj	2	2.49	1.25	0.03	0.97
	Residuals	24	1169.53	48.73		
Saturation						
	Urban_categorical	2	143.76	71.88	0.62	0.54
	Year_Adj	1	83.40	83.40	0.73	0.40
	Urban_categorical:Year_Adj	2	385.19	192.60	1.67	0.21
	Residuals	24	2760.89	115.04		
Brightness						
	Urban_categorical	2	348.87	174.43	5.22	0.01
	Year_Adj	1	93.07	93.07	2.78	0.11
	Urban_categorical:Year_Adj	2	2.36	1.18	0.04	0.97
	Residuals	24	802.05	33.42		
Face						
Hue						
	Urban_categorical	2	21.98	10.99	0.73	0.49
	Year_Adj	1	2.41	2.41	0.16	0.69
	Urban_categorical:Year_Adj	2	11.73	5.86	0.39	0.68
	Residuals	27	404.02	14.96		
Saturation						
	Urban_categorical	2	82.77	41.38	0.48	0.62
	Year_Adj	1	0.83	0.83	0.01	0.92
	Urban_categorical:Year_Adj	2	151.83	75.91	0.88	0.42
	Residuals	27	2319.80	85.92		

Brightness						
	Urban_categorical	2	44.40	22.20	0.36	0.70
	Year_Adj	1	124.20	124.20	2.04	0.16
	Urban_categorical:Year_Adj	2	9.78	4.89	0.08	0.92
	Residuals	27	1644.37	60.90		
Breast						
Hue						
	Urban_categorical	2	4.98	2.49	0.21	0.81
	Year_Adj	1	53.22	53.22	4.50	0.04
	Urban_categorical:Year_Adj	2	12.29	6.15	0.52	0.60
	Residuals	27	319.48	11.83		
Saturation						
	Urban_categorical	2	182.00	91.00	0.76	0.48
	Year_Adj	1	1.20	1.24	0.01	0.92
	Urban_categorical:Year_Adj	2	386.00	192.98	1.60	0.22
	Residuals	27	3251.00	120.41		
Brightness						
	Urban_categorical	2	39.57	19.79	0.58	0.57
	Year_Adj	1	218.79	218.79	6.36	0.02
	Urban_categorical:Year_Adj	2	0.50	0.25	0.01	0.99
	Residuals	27	928.20	34.38		

Table S10: Pyrrhuloxia Female Coloration Traits ANOVA Table

Patch/trait	Variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Crest						
Hue						
	Urban_categorical	1	70.01	70.01	0.76	0.40
	Year_Adj	1	2.32	2.32	0.03	0.88
	Urban_categorical:Year_Adj	1	2.19	2.19	0.02	0.88
	Residuals	15	1374.61	91.64		
Saturation						
	Urban_categorical	1	490.68	490.68	2.75	0.12
	Year_Adj	1	43.68	43.68	0.24	0.63
	Urban_categorical:Year_Adj	1	72.20	72.20	0.40	0.53
	Residuals	15	2679.08	178.61		
Brightness						
	Urban_categorical	1	6.51	6.51	0.12	0.73
	Year_Adj	1	140.53	140.53	2.67	0.12
	Urban_categorical:Year_Adj	1	76.39	76.39	1.45	0.25
	Residuals	15	789.87	52.66		
Face						
Hue						
	Urban_categorical	1	9.53	9.53	0.51	0.49
	Year_Adj	1	116.96	116.96	6.21	0.02
	Urban_categorical:Year_Adj	1	90.28	90.28	4.79	0.04
	Residuals	17	320.23	18.84		
Saturation						
	Urban_categorical	1	245.05	245.05	1.62	0.22
	Year_Adj	1	17.94	17.94	0.12	0.73
	Urban_categorical:Year_Adj	1	108.96	108.96	0.72	0.41
	Residuals	17	2570.05	151.18		

Brightness						
	Urban_categorical	1	0.14	0.14	0.00	0.95
	Year_Adj	1	200.12	200.12	4.80	0.04
	Urban_categorical:Year_Adj	1	5.58	5.58	0.13	0.72
	Residuals	17	708.47	41.68		
Breast						
Hue						
	Urban_categorical	1	15.75	15.75	0.9196	0.351026
	Year_Adj	1	267.918	267.918	15.6426	0.001022
	Urban_categorical:Year_Adj	1	0.167	0.167	0.0097	0.922536
	Residuals	17	291.166	17.127		
Saturation						
	Urban_categorical	1	283.95	283.95	5.44	0.03
	Year_Adj	1	232.01	232.01	4.45	0.05
	Urban_categorical:Year_Adj	1	60.48	60.48	1.16	0.30
	Residuals	17	886.79	52.16		
Brightness						
	Urban_categorical	1	0.22	0.22	0.01	0.94
	Year_Adj	1	441.69	441.69	10.67	0.00
	Urban_categorical:Year_Adj	1	4.43	4.43	0.11	0.75
	Residuals	17	703.45	41.38		

Table S11: Northern Cardinal Male Trait Sizes ANOVA Table

Trait	Variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Bill Length						
	Urban_categorical	2	0.95	0.47	0.25	0.78
	Tarsus	1	4.18	4.18	2.19	0.15
	Year_Adj	1	10.87	10.87	5.71	0.02
	Urban_categorical:Year_Adj	2	0.72	0.36	0.19	0.83
	Residuals	31	58.985	1.90		
Bill Width						
	Urban_categorical	2	1.50	0.75	2.84	0.07
	Tarsus	1	0.05	0.05	0.17	0.68
	Year_Adj	1	2.58	2.58	9.81	<0.01
	Urban_categorical:Year_Adj	2	0.88	0.44	1.67	0.20
	Residuals	31	8.15	0.26		
Crest Length						
	Urban_categorical	2	55.46	27.73	2.59	0.09
	Tarsus	1	33.22	33.22	3.10	0.09
	Year_Adj	1	11.85	11.85	1.11	0.30
	Urban_categorical:Year_Adj	2	39.38	19.69	1.84	0.18
	Residuals	30	321.66	10.72		
Tail Length						
	Urban_categorical	2	936.68	468.34	13.44	0.00
	Tarsus	1	0.14	0.14	0.00	0.95
	Year_Adj	1	765.76	765.76	21.97	0.00
	Urban_categorical:Year_Adj	2	201.16	100.58	2.89	0.07
	Residuals	30	1045.45	34.85		
Wing Length						
	Urban_categorical	2	24.35	12.17	0.94	0.40
	Tarsus	1	33.83	33.83	2.63	0.12

	Year_Adj	1	3.99	4.00	0.31	0.58
	Urban_categorical:Year_Adj	2	0.01	0.01	<0.01	1.00
	Residuals	31	399.39	12.88		

Table S12: Northern Cardinal Female Trait Sizes ANOVA Table

Trait	Variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Bill Length	**Bill Length**					
	Urban_categorical	2	4.47	2.24	1.52	0.30
	Tarsus	1	0.01	0.00	0.00	0.96
	Residuals	5	7.35	1.47		
Bill Width	**Bill Width**					
	Urban_categorical	2	0.32	0.16	0.45	0.66
	Tarsus	1	0.06	0.06	0.17	0.69
	Residuals	5	1.75	0.35		
Crest Length						
	Urban_categorical	2	17.02	8.51	1.36	0.34
	Tarsus	1	17.82	17.82	2.84	0.15
	Residuals	5	31.38	6.28		
Tail Length						
	Urban_categorical	2	8.70	4.35	0.06	0.95
	Tarsus	1	1.24	1.24	0.02	0.90
	Residuals	5	392.06	78.41		
Wing Length						
	Urban_categorical	2	15.86	7.93	4.36	0.08
	Tarsus	1	8.61	8.61	4.73	0.08
	Residuals	5	9.09	1.82		

Table S13: Pyrrhuloxia Male Trait Sizes ANOVA Table

Trait	Variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Bill Length						
	Urban_categorical	2	2.37	1.18	1.72	0.20
	Tarsus	1	0.01	0.01	0.01	0.92
	Year_Adj	1	1.94	1.94	2.82	0.10
	Urban_categorical:Year_Adj	2	4.30	2.15	3.12	0.06
	Residuals	27	18.59	0.69		
Bill Width						
	Urban_categorical	2	4.99	2.49	3.38	0.05
	Tarsus	1	0.00	0.00	0.00	0.99
	Year_Adj	1	6.61	6.61	8.95	0.01
	Urban_categorical:Year_Adj	2	1.29	0.64	0.87	0.43
	Residuals	27	19.95	0.74		
Crest Length						
	Urban_categorical	2	8.78	4.39	0.48	0.62
	Tarsus	1	5.61	5.61	0.62	0.44
	Year_Adj	1	7.88	7.87	0.87	0.36
	Urban_categorical:Year_Adj	2	1.65	0.82	0.09	0.91
	Residuals	25	227.59	9.10		
Tail Length						
	Urban_categorical	2	94.99	47.50	1.46	0.25
	Tarsus	1	12.66	12.66	0.39	0.54
	Year_Adj	1	226.39	226.39	6.98	0.01
	Urban_categorical:Year_Adj	2	124.82	62.41	1.92	0.17
	Residuals	23	745.87	32.43		
Wing Length						
	Urban_categorical	2	0.17	0.08	0.02	0.98
	Tarsus	1	16.42	16.42	2.94	0.10

	Year_Adj	1	0.78	0.78	0.14	0.71
	Urban_categorical:Year_Adj	2	8.74	4.37	0.78	0.47
	Residuals	27	150.96	5.59		

Table S14: Pyrrhuloxia Female Trait Sizes ANOVA Table

Trait	Variable	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Bill Length						
	Urban_categorical	1	0.06	0.06	0.06	0.81
	Tarsus	1	8.58	8.58	7.87	0.01
	Year_Adj	1	1.13	1.13	1.04	0.32
	Urban_categorical:Year_Adj	1	0.49	0.49	0.45	0.51
	Residuals	17	18.52	1.09		
Bill Width						
	Urban_categorical	1	0.09	0.09	0.22	0.64
	Tarsus	1	0.69	0.69	1.78	0.20
	Year_Adj	1	0.39	0.39	1.02	0.33
	Urban_categorical:Year_Adj	1	0.22	0.22	0.56	0.46
	Residuals	17	6.55	0.39		
Crest Length						
	Urban_categorical	1	6.27	6.27	0.65	0.43
	Tarsus	1	28.72	28.72	2.96	0.10
	Year_Adj	1	0.31	0.30	0.03	0.86
	Urban_categorical:Year_Adj	1	0.38	0.38	0.04	0.84
	Residuals	17	164.92	9.70		
Tail Length						
	Urban_categorical	1	123.14	123.14	2.26	0.15
	Tarsus	1	23.57	23.57	0.43	0.52
	Year_Adj	1	16.97	16.97	0.31	0.58
	Urban_categorical:Year_Adj	1	6.14	6.14	0.11	0.74
	Residuals	17	927.13	54.54		
Wing Length						
	Urban_categorical	1	15.08	15.08	3.34	0.09
	Tarsus	1	13.86	13.86	3.07	0.10

	Year_Adj	1	24.75	24.75	5.48	0.03
	Urban_categorical:Year_Adj	1	1.42	1.42	0.32	0.58
	Residuals	17	76.76	4.52		

APPENDIX D

CHAPTER 4 SUPPLEMENTARY TABLES AND FIGURES

Figures

Figure S1: Maps of sampling locations by species and urban category. Map A (top): Northern cardinal sampling locations. **Map B** (bottom): Pyrrhuloxia sampling locations. Rural birds represented by a red-orange pin and urban birds represented by a dark green pin.

Figure S2: Unrooted RAxML best tree of all samples using 32,437 genome-spanning SNPs.

All northern cardinal samples cluster on the left and all pyrrhuloxia samples cluster on the right, with no clades forming of urban or rural samples.

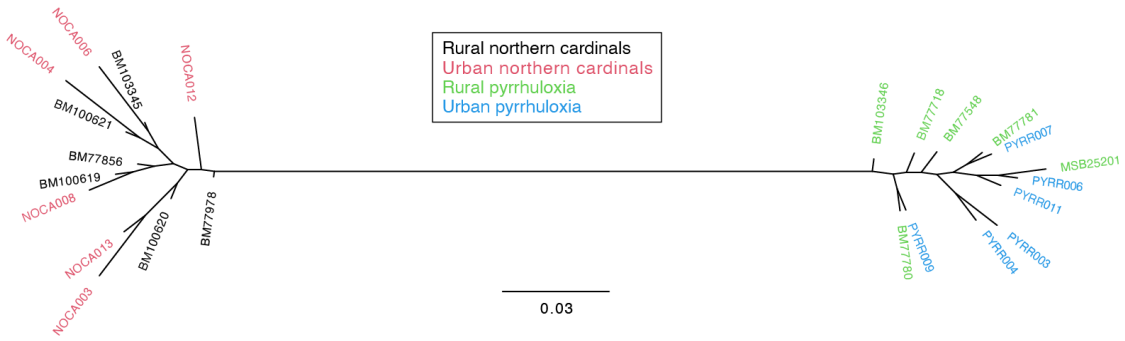


Figure S3: Principal Component Analyses.

Plot A (Left): PCA of all samples. PC 1 separates the two species (23.03% variation), but PC2 does not separate urban samples from rural samples. **Plot B** (Center): PCA of just northern cardinal samples. PC 1 mostly separates urban samples from rural samples (9.91% variation). **Plot C** (Right): PCA of just pyrrhuloxia samples. PC 1 separates urban samples from rural samples (23.03% variation)

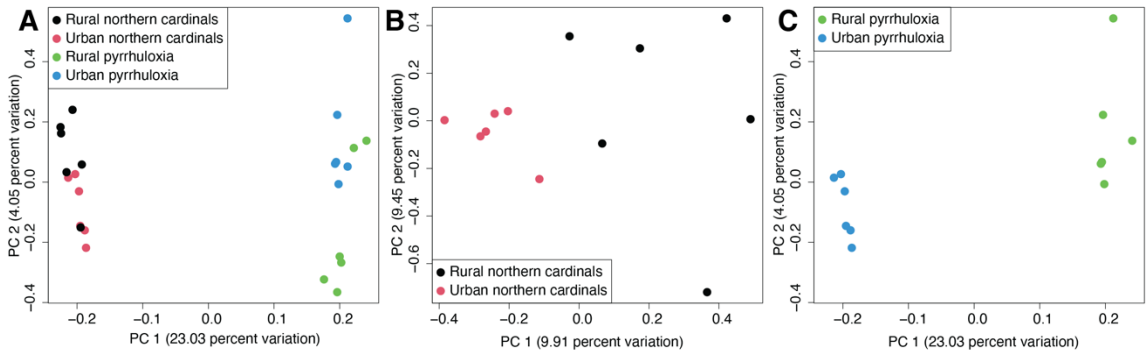


Figure S4: STRUCTURE plots (*K* 1-6).

All analyses regardless of the value used for *K* found separation by species but not by urban versus rural.

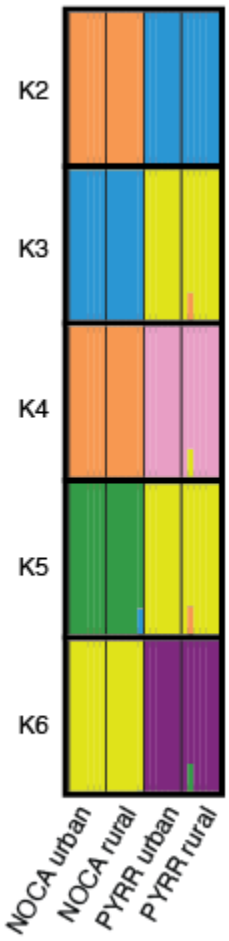
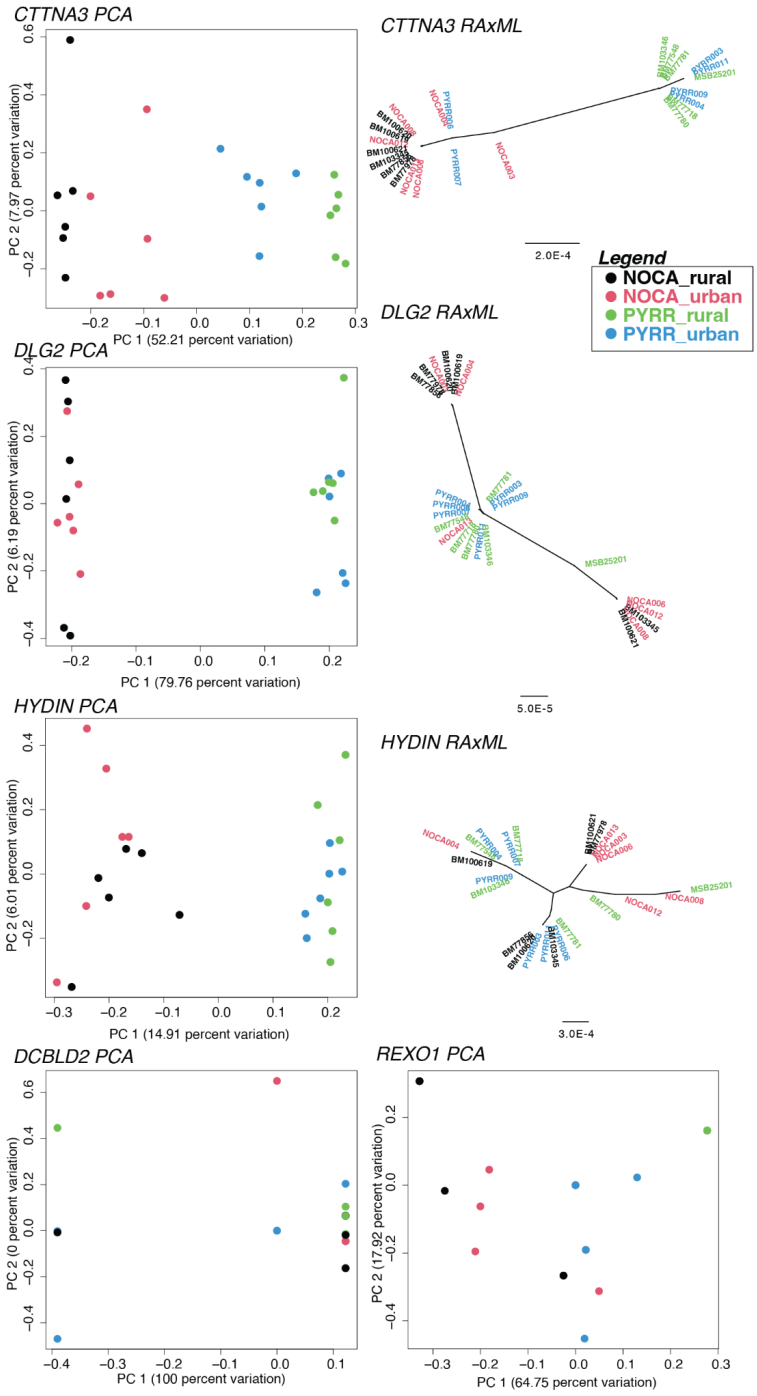


Figure S5: Gene Specific RAxML and PCA of genes that do not display patterns of introgression.

In all PCAs, PC1 separates the two species, and urban samples do not cluster. In all RAxML trees, either species cluster or no discernible pattern is apparent. CTNNA3 is the only outlier, as both the PCA and RAxML analysis are slightly consistent with some introgression, but could also be explained by convergent evolution in genotype.


CTTNA3 PCA
PC 2 (7.97 percent variation)
PC 1 (52.21 percent variation)
CTTNA3 RAxML
2.0E-4
Legend
NOCA_rural
NOCA_urban
PYRR_rural
PYRR_urban
DLG2 PCA
PC 2 (6.19 percent variation)
PC 1 (79.76 percent variation)
DLG2 RAxML
MSB25201
5.0E-5
HYDIN PCA
PC 2 (6.01 percent variation)
PC 1 (14.91 percent variation)
HYDIN RAxML
3.0E-4
DCBLD2 PCA
PC 2 (0 percent variation)
PC 1 (100 percent variation)
REXO1 PCA
PC 2 (17.92 percent variation)
PC 1 (64.75 percent variation)

Tables

Table S1: Regions of Interest

NOCA_Urban	NOCA_Rural	PYRR_Urban	PYRR_Rural	FST	TajimasD	NucleotideDiversity	SweeD	OmegaPlus	Scaffold	TrueMin	TrueMax	SearchMin	SearchMax	Genes
X	X	X	X	X					1005221	2174717	2174761	2124717	2224761	Plekhf2,Ch037
X	X			X					1008768	38618	38684	0	88684	
X	X			X					1009024	271029	371029	221029	421029	Ctnna3_0, Lrrtm3
X	X			X					1011506	97212	97217	47212	147217	Mrps5
X	X			X					1018132	45204	45210	0	95210	
X	X			X					1019412	27631	27631	0	77631	
		X	X	X					1006626	142206	142346	92206	192346	Ift122,Rho
		X	X	X					1008328	194860	194882	144860	244882	Tmcc1
		X	X	X					1008382	691075	791075	641075	841075	Fxr1_0,Fxr1_1
		X	X	X					1020886	118926	119094	68926	169094	Pcbp3_0,Col6a1
		X	X	X					1021115	168762	238957	118762	288957	Chga,Itpk1
X		X			X				1009321	580000	600000	530000	650000	Dlg2
X					X				1016584	0	20000	-50000	70000	Rexo1
	X				X				1007098	0	20000	-50000	70000	Mtco2_0,Kcna6
	X				X				1011537	0	20000	-50000	70000	Tp73,Tp73as1, Ccdc27,Lrrc47, Cep104,Dffb
	X				X				1019264	160000	180000	110000	230000	Slc26a5
	X				X				1026539	0	20000	-50000	70000	
		X			X				1000233	0	20000	-50000	70000	

		X			X				1001023	0	20000	-50000	70000	
		X			X				1002912	20000	40000	-30000	90000	Dcbld2
			X		X				1001302	0	20000	-50000	70000	
			X		X				1007741	620000	640000	570000	690000	Rad21
			X		X				1010057	0	20000	-50000	70000	
			X		X				1010419	20000	40000	-30000	90000	
			X		X				1010703	0	20000	-50000	70000	
			X		X				1019758	0	20000	-50000	70000	
			X		X				1019804	280000	300000	230000	350000	Kcnq3,Oc90,Efr3a
X	X	X	X			X			1006187	20000	40000	-30000	90000	Hydin_0
X		X					X		1006187	198	5561	-49802	55561	Hydin_0
	X						X		1023961	1073	2405	-48927	52405	
			X				X		1022801	5198	5593	-44802	55593	
X								X	1013494	364	442	-49636	50442	
	X							X	1017116	2124	2139	-47876	52139	
	X							X	1023961	4638	12327	-45362	62327	
		X						X	1008477	966	1218	-49034	51218	
			X					X	1015323	152	1532	-49848	51532	