SEXUAL SELECTION AND SPECIATION IN THE RED-BACKED FAIRY-WREN

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A longstanding question in evolutionary biology is whether divergence in sexual signals leads to speciation. A powerful approach is to examine closely related taxa that appear to be in the process of speciation, incorporating ecological, behavioral, morphological, and genetic data to understand how sexual selection affects the speciation process. The red-backed fairy-wren (*Malurus melanocephalus*) is a small passerine bird endemic to Australia that is classified as two subspecies based primarily on variation in two sexual traits: red vs. orange male nuptial plumage color and tail length. My dissertation focused on analyzing the pattern of genetic and morphological variation between the two subspecies and conducting field experiments to explore the selective forces and behavioral mechanisms responsible. Spatial modeling analyses revealed that the two subspecies exhibited variation in numerous non-sexual traits that was well explained by underlying environmental variation. In stark contrast, variation in plumage color could not be explained by environment alone, but rather exhibited a clear pattern of isolation by distance, suggesting it has diverged stochastically as a result of divergent Fisherian sexual selection. I then compared clinal variation in allele frequencies at many unlinked single nucleotide polymorphism (SNP) loci to variation in plumage color across a 3,052 km transect through the species range. Clines for many SNP loci were centered at the Carpentarian Barrier, confirming the existence of a hybrid zone at the location of secondary contact between the subspecies. The cline for plumage color was displaced 390 km east of the genetic clines, indicating that alleles for red plumage have introgressed asymmetrically across the hybrid zone, likely driven by sexual selection. A plumage manipulation experiment in an allopatric population of the orange subspecies confirmed

this idea, as experimentally reddened males sired significantly more extra-pair young and had higher reproductive success than orange males. To determine whether this mating advantage was due to female preference or a competitive advantage of red over orange males, I presented territorial males in populations on both sides of the hybrid zone with various combinations of local, foreign, and heterospecific male mounts paired with local, foreign, and heterospecific songs. Territorial males consistently responded most aggressively to the local song regardless of mount plumage color, suggesting that plumage color is not a signal used in male competition. Thus, female preference for red, and not a competitive advantage to red males, likely drives the asymmetrical introgression of red plumage. In conclusion, although the red-backed fairy-wren appeared at first to be in the process of speciation by sexual selection owing to the conspicuous divergence in sexual signals between the subspecies, a thorough analysis of the system revealed a much more complicated situation. This study highlights the strength of fine-scale analyses using behavioral experiments to elucidate broader patterns of speciation. Divergence in sexual signals, as seen here, will not always lead to reproductive isolation and speciation, particularly if response to those signals has not diverged in tandem. In the red-backed fairy-wren, sexual selection instead appears to have eroded one potential speciation phenotype between subspecies. This phenomenon may be more common in species at an intermediate stage of divergence, particularly those subject to strong sexual selection that exhibit alternative mating tactics such as extra-pair mating.

BIOGRAPHICAL SKETCH

Daniel Timothy Baldassarre was born on July 22, 1986 in Opelika, Alabama to Guy and Eileen Baldassarre. Guy was then a professor of ornithology and wildlife science at Auburn University, but soon got a job at SUNY College of Environmental Science and Forestry in Syracuse, NY, so the family moved there when Dan was a year old. They settled in the small town of Tully, where Dan spent the rest of his youth. Every summer, the family lived at the Cranberry Lake Biological Station in the Adirondacks, where Guy taught field courses, and Dan spent every day terrifying his mother by speeding down rutted dirt tracks on his bike, playing in poison ivy, and having rock fights in the gravel pit with his brother, Adam. When not raising hell, it was here that Dan also got his first real exposure to science. There were mammalogists trapping squirrels, entomologists tracking honeybees, and ornithologists mist-netting barn swallows all around him. Slowly but surely, Dan was subtly indoctrinated by the cult that is biological research. For the longest time, he wanted to be an entomologist, and amassed an impressive insect collection. Thankfully, he eventually saw the light, and decided he was more interested in birds. As an eight-year-old, he spent nine months with his family on the Yucatán Peninsula in Mexico while his dad was on sabbatical studying flamingos. There he spent enough time outside for his normally brown hair to become thoroughly sun-bleached, and enough time watching his dad conduct fieldwork to convince himself that he wanted to spend the rest of his life learning about nature. After narrowly escaping the twisted disciplinary power structure of Tully High School (at one point the principal accused him of being a terrorist), Dan enrolled as a biology major at Syracuse University in 2004. There he had the great fortune of being mentored by Drs. Larry Wolf, Scott Pitnick, and Al Uy; and Drs. Scott Turner and Bill Shields at nearby SUNY ESF. It was at this time, especially after conducting an undergraduate research project in Dr. Uy's lab, that he began to realize that he could actually translate his love of nature and science into some sort of semi-respectable career. After lots of good advice and experience working on field projects in Alaska, Long Island, Namibia, and Costa Rica, he settled on a graduate program at

Washington State University under the guidance of Dr. Mike Webster. Mike asked Dan how he would feel about working on fairy-wrens in Australia, and Dan decided that sounded like fun. It turned out to be an incredible experience where he got to travel throughout the outback dodging bush fires and biblical flooding, all the while delving deep into the everyday lives of these magnificent little birds. After transferring to Cornell in 2009, he managed to cobble together a research project examining the effect of their mating behavior on the speciation process, combining five years of field work and hundreds of lab hours doing genetic analyses. He had an absolute blast in grad school. Dan hopes to continue his love of behavioral ecology and ornithology as a postdoctoral researcher, and eventually a university-level professor. His goal is to always be able to say, in the words of his dad, "As long as I've 'worked' with birds, I've never really had to work at all. All true ornithologists understand that statement".

Dedicated to my dad, Guy Baldassarre (1953-2012).

We never got to write a “Baldassarre and Baldassarre” paper together,

but I think you would have been proud of this.

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PREFACE

Many closely related species exhibit conspicuous variation in secondary sexual characteristics, suggesting that sexual selection often drives divergence and speciation. Most research focusing on sexual selection and speciation takes a comparative approach, examining phylogenies at a broad scale and testing for associations between sexual trait diversification and speciation rate. However, these studies have limited power to explore the underlying ecological and behavioral mechanisms driving these patterns. A more powerful approach is to examine closely related taxa that appear to be in the process of speciation, incorporating ecological, behavioral, morphological, and genetic data to understand how sexual selection affects the speciation process.

The red-backed fairy-wren (*Malurus melanocephalus*) is a small passerine bird endemic to Australia that is classified as two subspecies based primarily on variation in two sexual traits: male nuptial plumage color and tail length. The short-tailed, red *M. m. cruentatus* subspecies occurs in western and northern Australia, and the long-tailed, orange *M. m. melanocephalus* subspecies occurs along the east coast, with a zone of overlap in northeast Queensland. Previous research has indicated that the two subspecies are moderately genetically differentiated across the Carpentarian Barrier, a prominent biogeographic barrier in northwest Queensland that experienced widespread aridity during the Pleistocene, forcing the subspecies into allopatric refugia in the west and southeast. The current continuous distribution of the subspecies across this barrier thus suggests that there is a hybrid zone located in this area that resulted from secondary contact. My dissertation is broadly divided into two sections: in Chapters 1 and 2, I analyzed the pattern of genetic and morphological variation between the two subspecies, and in Chapters 3 and 4, I conducted two field experiments to explore the selective forces and behavioral mechanisms responsible for the observed patterns.

In Chapter 1, I used the spatial modeling technique generalized dissimilarity modeling (GDM) to quantify variation in sexual and non-sexual morphological traits across the species

range. This technique compares the relative influence of bioclimatic environmental variables (e.g., temperature, rainfall, vegetation) and geographic distance on phenotypic variation. GDM analyses revealed that the two subspecies exhibited variation in numerous non-sexual traits (e.g., weight, wing length, tarsus length) that was well explained by underlying environmental variation, but was not broadly diagnostic of the two subspecies. One putative sexual signal, tail length, exhibited a similar pattern, but with a small influence of geographic distance, suggesting it has diverged stochastically to some extent, but is also constrained by the environment. In stark contrast, variation in plumage color (quantified by reflectance spectrophotometry) could not be explained by environment alone, but rather exhibited a clear pattern of isolation by distance, suggesting it has diverged stochastically as a result of divergent Fisherian sexual selection while the subspecies were geographically isolated. In addition, there was no appreciable divergence in any morphological traits across the genetic barrier in northwest Queensland, while divergence in plumage color was most pronounced across the plumage contact zone in northeast Queensland. This pattern suggests that alleles for red plumage may have introgressed eastward across the hybrid zone following secondary contact.

In Chapter 2, to more explicitly test the hypothesis of asymmetrical introgression of red plumage, I used genomic and plumage color data in a geographic cline analysis of the hybrid zone. I used the reduced representation genomic technique genotyping-by-sequencing to create a large, genome-wide single nucleotide polymorphism (SNP) database. I compared variation in allele frequencies at these SNP loci to variation in plumage color across a 3,052 km transect through the hybrid zone encompassing nearly the entire species range. Clines for many putatively unlinked SNP loci were narrow relative to dispersal and centered at the Carpentarian Barrier, confirming that it is the location of secondary contact between the subspecies. However, the center and width of these genetic clines varied substantially, with some introgressing freely and others subject to weak selection, suggesting the hybrid zone is a semi-permeable tension zone. The cline for plumage color was displaced 390 km east of the genetic clines, indicating that alleles for red plumage have introgressed asymmetrically across the hybrid zone, likely driven by

sexual selection.

In Chapter 3, I conducted a plumage manipulation experiment in an allopatric population of the orange subspecies to test the hypothesis that sexual selection on red plumage in this area has driven the observed asymmetrical introgression of red plumage. The experiment consisted of three groups of males that were captured at the beginning of the breeding season: control males that were not manipulated, sham males that were painted with a non-toxic colorless marker, and reddened males that were painted with a non-toxic red marker. These males were then released into the population, and their subsequent mating behavior was monitored. Reflectance spectrophotometry confirmed that the colorless marker did not alter the natural orange color, whereas the red marker accurately mimicked the natural color of the western subspecies. Experimental groups did not differ in the number of within-pair young sired or in the likelihood that they would be cuckolded. However, reddened males sired significantly more extra-pair young, resulting in higher reproductive success than control or sham males. These results suggest that female preference for red males as extra-pair mates drives the asymmetrical introgression of red plumage. Furthermore, this preference for red is likely due to a sensory bias, as females did not exhibit a preference for naturally occurring red males, only those that were manipulated to exhibit the phenotype of the foreign subspecies.

Finally, in Chapter 4 I conducted a mount presentation/song playback experiment to more rigorously test the hypothesis that the increased reproductive success of red males in an otherwise orange population – and the resulting introgression of red plumage – is due to female preference and not a competitive advantage of red over orange males. For this experiment, I presented territorial males with various combinations of local, foreign, and heterospecific male mounts paired with local, foreign, and heterospecific songs. This experimental protocol was replicated in allopatric populations on both sides of the hybrid zone. Results were similar in both populations: territorial males consistently responded most aggressively to the local song regardless of mount plumage color, and there was no asymmetry between populations in response strength or behaviors. These results suggest that song is used as an intrasexual signal

between males during social competition, and may function as a speciation phenotype in the hybrid zone. In contrast, male plumage color does not appear to be used by males as a signal during competition, corroborating the conclusion of the plumage manipulation experiment that it is an intersexual signal used by females to evaluate potential extra-pair mates. Thus, female preference for red, and not a competitive advantage to red males, likely drives the asymmetrical introgression of red plumage.

In conclusion, although the red-backed fairy-wren appeared at first to be in the process of speciation by sexual selection owing to the conspicuous divergence in sexual signals between the subspecies, a thorough analysis of the system revealed a much more complicated situation. The two subspecies do indeed differ in sexual signals, particularly red vs. orange male nuptial plumage color, and this divergence was likely driven by divergent sexual selection in the absence of strong environmental influence. Genomic analyses revealed the existence of a hybrid zone that resulted from secondary contact at the Carpentarian Barrier, but the plumage contact zone is displaced several hundred kilometers into the range of the orange subspecies, suggesting asymmetrical introgression of alleles for red plumage. This introgression was likely facilitated by strong sexual selection on red males dispersing into orange populations, as experimentally reddened males sired significantly more extra-pair young and had higher reproductive success than orange males. Furthermore, this mating advantage appears to be due to female mate choice and not male competition, as aggressive response of territorial males was not influenced by plumage color. This study highlights the strength of fine-scale analyses of taxonomic pairs that incorporate data from behavioral experiments to elucidate broader patterns of speciation. Divergence in sexual signals, as seen here, will not always lead to reproductive isolation and speciation, particularly if receiver response to those signals has not diverged in tandem. In the red-backed fairy-wren, sexual selection instead appears to have eroded one potential barrier to gene flow between subspecies. This phenomenon may be more common in species at an intermediate stage of divergence, particularly those subject to strong sexual selection that exhibit alternative mating tactics such as extra-pair mating.

CHAPTER 1

THE ROLE OF ECOLOGICAL VARIATION IN DRIVING DIVERGENCE OF SEXUAL AND NON-SEXUAL TRAITS IN THE RED-BACKED FAIRY-WREN

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Abstract

Many species exhibit geographic variation in sexual signals, and divergence in these traits may lead to speciation. Sexual signals may diverge due to differences in ecology if the environment constrains signal production or transmission. Alternatively, sexual signals may diverge stochastically through sexual selection or genetic drift, with little environmental influence. To distinguish between these alternatives I quantified variation in two putative sexual signals – tail length and plumage color – and a suite of non-sexual morphometric traits across the geographic range of the red-backed fairy-wren (*Malurus melanocephalus*). I then tested for associations between these traits and a number of environmental variables using generalized dissimilarity models. Variation in morphometric traits was well explained by environmental variation, irrespective of geographic distance between sites. Among putative signals, variation in plumage color was best explained by geographic distance, whereas tail length was best explained by environmental variation. Divergence in male plumage color was not coincident with the boundary between genetic lineages, but was greatest across a contact zone located approximately 300 km east of the genetic boundary. Morphometric traits describing size and shape have likely been subject to ecological selection and thus appear to track local environmental variation regardless of subspecies identity. Ecological selection appears to have also influenced the evolution of tail length as a signal, but has played a limited role in shaping geographic variation in plumage colora, consistent with stochastic divergence in concert with Fisherian selection on this trait. The lack of coincidence between the genetic boundary and the contact zone between plumage types suggests that the sexual plumage signal of one subspecies has introgressed into the genetic background of the other. Thus, this study provides insight into the various ways in which signal evolution may occur within a species, and the geographic patterns of signal variation that can arise, especially following secondary contact.

Introduction

Divergence in sexual signals is important to the generation of biodiversity because it may lead directly to assortative mating and reproductive isolation between taxa (Coyne and Orr 2004). Indeed, because sexual signals affect mate recognition and thus pre-mating isolation, their divergence may lead to reproductive isolation more rapidly than the accumulation of post-zygotic barriers (Turelli et al. 2001; Coyne and Orr 2004). However, the relative importance of different evolutionary forces in causing variation and divergence of sexual signals remains unclear (Shaw and Mullen 2011).

Non-sexual traits that are of direct ecological importance, such as those affecting foraging and thermoregulation, are likely to diverge via ecological selection and therefore should track underlying environmental variation (Schluter 2001). For example, variation in bill length is correlated with environmental differences between the Andean lowlands and highlands in the speckled hummingbird (*Adelomyia melanogenys*), most likely due to different selection regimes imposed by variable flower morphologies (Chaves et al. 2007). Similarly, sexual signals may be constrained by ecology and environmental variation if, for example, diet affects the expression of the trait, (e.g., Hill 1992), or if ecological factors constrain the transmission of signals (e.g., Marchetti 1993). In these situations, ecological selection helps shape the sexual signal, and geographic variation in the signal should also track environmental variation.

Alternatively, sexual signals may diverge via stochastic processes independent of environmental variation (Lande 1981; Uyeda et al. 2009). In these models, random drift in the responses of receivers to variation in sexual signals can cause rapid and stochastic change in the signal, leading to phenotypic variation across populations that is not correlated with ecological factors. For example, Prum (1997) found that the explosive radiation of sexual signals in Neotropical manakins (Family: Pipridae) is consistent with a stochastic divergence followed by Fisherian sexual selection with little direct or indirect influence of ecology. There are thus multiple phenotypic axes along which taxa may diverge during speciation, with varying opportunities for environmental influence. The relative importance of ecological selection

compared to Fisherian sexual selection, especially during early divergence of sexual signals, is the subject of recent debate (see Prum 2010).

I examined these issues in the red-backed fairy-wren (*Malurus melanocephalus*), a small insectivorous passerine bird endemic to Australia. Currently there are two recognized subspecies that differ primarily in male plumage color and tail length (Rowley and Russell 1997): the crimson-backed, shorter-tailed *M. m. cruentatus* subspecies occurs in northern Australia (Figure 1.1b), and the orange-backed, longer-tailed *M. m. melanocephalus* subspecies occurs in eastern Australia (Figure 1.1c). These subspecies are thought to also differ somewhat in morphometric traits, for example, *M. m. cruentatus* has been described as weighing less than *M. m. melanocephalus* (Rowley and Russell 1997). Both subspecies are found in qualitatively similar open tropical savannah habitats across their entire range. There is a relatively large morphological contact zone between these subspecies in northern Queensland that is defined subjectively by the presence of males with intermediate values for tail length and plumage color (Rowley and Russell 1997). Previous work has shown that male plumage color is a carotenoid-based (Rowe and McGraw 2008) intersexual signal used by females during mate choice (Karubian 2002; Webster et al. 2008), and male tail length appears to be an intrasexual signal used primarily during male competitive interactions (Karubian et al. 2009). The two subspecies are moderately genetically differentiated across the Carpentarian Barrier, a well-known biogeographic barrier (Cracraft 1986), suggesting divergence during the Pleistocene (Lee and Edwards 2008), likely followed by secondary contact.

Because the red-backed fairy-wren is at an early stage of speciation and exhibits variation in both sexual and non-sexual traits, it is an excellent species in which to explore the effects of the environment on divergence in sexual signals. In this study, I aimed to do this by analyzing variation in a suite of sexual and non-sexual traits across the species range and determining which traits are related to underlying environmental variation. Traits subject to ecological selection (such as morphometric traits that influence feeding behavior or locomotion) should correlate with variation in the physical environment. In contrast, traits subject to stochastic

divergence in concert with sexual selection should not be correlated with environmental variation, but should instead exhibit geographic variation that is better explained by geographic distance, with greater distance signifying more time to accumulate stochastic variation (i.e., isolation by distance, Wright 1943). By comparing the effects of environmental variables and geographic distance on sexual signals and morphometric traits, I aimed to distinguish between two alternative hypotheses: 1) if sexual signals have evolved via ecological selection, variation in these traits should be correlated with environmental variables, as is predicted for non-sexual morphometric traits; 2) in contrast, if sexual signals have evolved via sexual selection coupled with genetic drift, then this variation should be better explained by geographic distance, independent of environment. In addition, to further explore the geographic patterns of trait divergence between subspecies, I examined the influence of the Carpentarian Barrier and the plumage contact zone that exists further east (Figure 1.1a) on trait dissimilarity (see methods for quantification of the eastern contact zone). Traits that have diverged between the two genetic lineages should show a strong signal of differentiation across the Carpentarian Barrier, as it is the hypothesized location of secondary contact and the genetic boundary between the subspecies (Lee and Edwards 2008).

Methods

Field methods

Between 2004 and 2011, colleagues and I captured 480 adult red-backed fairy-wrens at 24 sites throughout their range (Figure 1.1a). We captured birds using mist nets, weighed them to the nearest tenth of a gram using a spring scale, and measured the following morphometric traits to the nearest tenth of a millimeter with digital calipers: wing length, tarsus length, tail length, exposed culmen length (the length from the tip of the bill to the middle of the nares), bill width and depth (measured at the middle of the nares), and the total length of the bill plus head (distance from the back of the head to the tip of the bill). Feather samples from the center of the back (which are colored red-orange) were taken from all adult males in breeding plumage (N =

241) for color quantification using reflectance spectrophotometry. The plumage color and male tail length data set was restricted to 21 sites, because no males in breeding plumage were captured at three sites (Figure 1.1a). In my analyses of non-sexual morphometric traits, "tail" refers to tail length of males and females combined, and includes birds from all 24 sites.

Reflectance spectrophotometry and plumage color analysis

I used reflectance spectrophotometry to objectively measure plumage color variation. From each individual male's feather sample, I mounted six feathers in an overlapping pattern on a square of black construction paper (Strathmore Artagain® Coal Black). To measure reflectance of the feather sample, I used an Ocean Optics USB2000 UV-VIS spectrometer with a R200-7 UV-VIS probe, and a PX2 pulsed xenon light source. The probe tip was mounted in a metal block to exclude ambient light, and the probe illuminated a measurement area of 3 mm^2 on the feather sample. Three reflectance curves were generated for each sample, and the probe was re-calibrated against a white standard (Ocean Optics WS-1) after each individual. I averaged the three reflectance curves to produce one curve per individual, and the reflectance in the avian visible spectrum (300 nm-700 nm) was analyzed.

To obtain color metrics accounting for the spectral sensitivity of the avian visual system, I used the program TetraColorSpace (Stoddard and Prum 2008), which analyzes reflectance curves using the spectral sensitivity of each of the cones in the avian retina, and plots each color as a point in a tetrahedral color space. The red-backed fairy-wren has a violet-sensitive visual system (Odeen et al. 2012) so I analyzed feather samples using the average avian violet-sensitive spectral sensitivity curve (Endler and Mielke 2005). For the present analysis, I used the color metric θ (theta), which describes plumage hue. Theta is defined as the angular displacement of the color vector from the positive x-axis of the tetrahedral color space, which runs between the green and red vertices (Stoddard and Prum 2008). I chose this metric because it best captures the geographic variation in plumage color (i.e., there are no consistent differences in plumage brightness or chroma across the species range, unpublished data). Theta can be interpreted as the

direction of the color vector (Stoddard and Prum 2008), and quantifies, in this case, orange vs. red plumage.

Delineation of habitat range using species distribution modeling

To delineate a suitable habitat range for the red-backed fairy-wren, I used the "maximum entropy" species distribution modeling approach employed in the program Maxent 3.0 (Phillips et al. 2006). Maxent uses presence only data plus environmental variables at sites where the species has been recorded. I used 6,865 localities of known species occurrences, from an initial total of 18,595 records from BirdLife Australia (www.birdata.com.au/maps.vm); the initial dataset was reduced by only including a single known locality per 30 x 30 arcsec grid cell. Maxent returns logistic probabilities for each grid cell, with increasing values indicating higher probabilities of species occurrence. These values were then converted to a presence/absence map using the threshold "balance training omission, predicted area and threshold value" (Phillips et al. 2006). I used the following settings in Maxent: 1,000 background points, auto features, regularization multiplier = 1.0, maximum iterations = 500, convergence threshold = 0.00005. Because I was mainly interested in a relatively broad delineation of the distribution of the red-backed fairy-wren, rather than identifying the limiting conditions of their distributions or the distributions of sub-species, I did not perform further tests of the Maxent model. This will be the subject of a future study. The resulting model had an AUC (area under the receiving operator curve, Phillips et al. 2006) value of 0.77, and describes an area of suitable habitat consisting mainly of open tropical savannah (Figure 1.2). The model is highly consistent with the previously estimated species range of the red-backed fairy-wren (Rowley and Russell 1997).

Predictor variables included in models

In an attempt to capture biologically meaningful variation in the physical environment across the range of the red-backed fairy-wren, I included data from several ground-based and remotely sensed sources (Table 1.1). Eleven bioclimatic variables from the WorldClim database

(www.worldclim.org) were included that represent annual means, seasonality, and seasonal extremes in temperature and rainfall (Hijmans et al. 2005). These data were collected from weather stations and represent climatic variation between 1950-2000 that has been shown to accurately characterize the species ranges of Australian taxa (Nix 1986). In addition to these bioclimatic variables, I also included a suite of remotely sensed environmental variables in my analyses. The QuickScat product (www.scp.byu.edu) uses active radar scatterometers to measure surface moisture and is insensitive to cloud cover. High-resolution elevation data was gathered from the Shuttle Radar Topography Mission (SRTM). Finally, from the moderate resolution imaging spectroradiometer aboard the MODIS satellites (www.modis.gsfc.nasa.gov), I included data on percent tree cover and photosynthetic activity as measured by the normalized difference vegetation index (NDVI). To enable simultaneous comparison of the effects of all environmental variables, those with resolutions higher (e.g., SRTM, 30 m) or lower (e.g., QuickScat, 2.25 km) than 1 km were re-aggregated to a 1 km grid cell resolution. Geographic distance was computed as the shortest straight-line distance between two sampling sites.

To examine the importance of the Carpentarian Barrier and the eastern contact zone in explaining trait divergence, I introduced a binary GIS layer for each region as a predictor variable in the full model, with values equal to 0 west of the region and 1 east of the region. Thus, comparisons between sites across the region receive a value of 1 for this predictor variable, while comparisons between sites on the same side of the region receive a value of 0. Preliminary analysis of variation in plumage hue across the red-backed fairy-wren range suggested that the eastern contact zone between plumage types is in fact located further down the east coast than is suggested by the previously published species range (Figure 1.3). Thus, I used the area defined by the approximate center of the plumage cline as the eastern contact zone in my models. More rigorous cline-based analyses of spatial variation in plumage hue are the subject of Chapter 2.

Generalized dissimilarity modeling of phenotypic variation

To analyze the effect of environmental variation on phenotypic variation across the red-backed

fairy-wren range, I used generalized dissimilarity modeling (GDM, Ferrier et al. 2007). GDM is a matrix regression technique that predicts biotic dissimilarity across the landscape based on the matrix correlation between biotic dissimilarity and environmental dissimilarity plus geographic distance between sites where the species has been sampled. GDM can fit non-linear relationships between environmental variables and phenotypic traits using I-spline basis functions, and also can consider the effect of geographic distance independent of environmental variables. This is an important feature of GDM that controls for the often-observed correlation between geographic distance and environmental dissimilarity by analyzing the independent effect of distance. GDM is a two-step process. First, dissimilarities in predictor variables (i.e., environmental variables, geographic distance, and potential isolating barriers) between all pairwise combinations of sampling sites are fit to dissimilarities in response variables (i.e., phenotypic traits). For all phenotypic traits, I computed dissimilarity between sites as the Euclidian distance between the average trait values at each site divided by the sum of the standard deviation at each site, to control for within-site variability. The relative contribution of each predictor variable is tested using a permutation with the following structure: the predictor variables are introduced to the model in random order and the variation in the response variable explained by the inclusion of that variable is compared to that without the variable (ΔD). Next, the predictor variable is added again in a large number of random permutations of the order of sampling locations, resulting in a random distribution of ΔD (Ferrier et al. 2007). ΔD resulting from the inclusion of the properly seeded response variable is compared to that distribution, and the variable is retained if ΔD is significantly higher than the random distribution. Second, using these results, a spatial pattern of response variables is predicted across the entire range as selected by the Maxent species distribution model described above. This pattern is color-coded, with differences in color proportional to differences in the response variable. The relative importance of each predictor variable can be assessed by response curves, where the maximum height is indicative of the relative importance, and the slope indicates the predicted rate of change in the response variable as a function of the predictor variable. I considered predictors retained in the model particularly

important if they exhibited a response curve with a height ≥ 50% of the predictor with the highest overall response (Thomassen et al. 2010).

To consider the possibility of a correlation between geographic distance and environmental dissimilarity, I ran three separate models for each response variable with the following predictor variables: a full model with environmental variables, geographic distance, and potential isolating barriers; a model with only environmental variables; and a model with only geographic distance. To examine the fit of the models compared to the null hypothesis of no influence of any predictor variable, I also ran a model with random values of environmental variables in each grid cell and the variation explained by the full model was compared to the random model (Thomassen et al. 2010). Computational limitations prevented the creation of a distribution of random models for each trait, but the difference in explanatory power between the random and full models was quite large (typically an order of magnitude, see results), thus I felt confident evaluating the strength of the models compared to one iteration of the random model.

Results

Morphometric variation

For all morphometric traits, the full model explained much more variation in the data (mean = 32.1%, SD = 15.4, range= 11.7-56.5%, Table 1.2) than did the associated random model, (*t*-test comparing mean percent variation explained by full models and random models: $t = 5.08$, df = 7.59, $p = 0.001$). In all cases, the distance-only model was similar to the random model, with a low percent variation explained (mean = 3.45%, SD = 3.7, range = 0.9-11.5%). For all but two morphometric traits, neither geographic distance nor either potential barrier was retained as an important predictor in the full model, and the environment-only and full models were thus identical. The full model for wing length did include geographic distance and the Carpentarian Barrier as important predictors, but they contributed relatively little to the fit of the model, improving the overall fit by only 0.7%. Similarly, the full model for bill width included the eastern contact zone as an important predictor, but it improved the overall fit by only 0.5%.

For each morphometric trait, different combinations of environmental variables were retained as important predictors, resulting in markedly different patterns of spatial variation (see Figures 1.4a and 1.4b for examples of predicted variation in morphometric traits analyzed). Although many morphometric traits were influenced by a combination of environmental variables considered important based on the height of their response curves (numbers in parentheses in Table 1.2), there were two models where a single environmental variable exhibited a response curve dramatically higher than any other. In the models of weight and tarsus length, this single environmental variable appeared to drive the overall pattern in each case: there was a strong negative relationship between weight and Bio1: mean temperature (linear regression, $r^2 = 0.70$, $p < 0.001$, Figure 1.5a), and a strong positive relationship between tarsus length and percent tree cover (linear regression, $r^2 = 0.41$, $p < 0.001$, Figure 1.5b). Finally, environmental variation explained significantly less variation in bill morphometric traits (culmen, width, and depth) than in other body morphometric traits (*t*-test comparing mean percent variation explained by GDM models of bill and non-bill morphometrics: $t = 2.57$, df = 5, $p = 0.001$).

Plumage hue variation

All models for plumage hue performed substantially better than the random model (Table 1.2). The distance-only model explained a substantial proportion of the variation in plumage hue (47.6%), and this variation explained was much greater than the variation explained by distance for any of the morphometric traits (mean = 3.45 ± 3.7%). The environment-only model also explained a substantial amount of variation (62.6%), but this pattern appeared to be driven by a spatial correlation between geographic distance and environmental dissimilarity. A linear regression of the most important predictor in the environment-only model (Bio15: precipitation seasonality) on geographic distance supports this idea ($r^2 = 0.24$, $p < 0.001$, Figure 1.6). Overall, the best fitting model for plumage hue also included the eastern contact zone as a predictor variable, and it improved the fit of the full model from 69.3% to 78.9%. In addition to the

improvement of the fit by adding the eastern contact zone, the independent effect of geographic distance was the most important predictor variable in the full model (Figures 1.4c and 1.7a). In contrast, the Carpentarian Barrier – the boundary between genetic lineages – was not selected as an important predictor of plumage hue in any model.

Male tail length variation

All models for male tail length performed better than the random model (Table 1.2). The distance-only model explained more variation in male tail length (13.5%) than in any other morphometric trait, although not as much as in plumage hue (see above). The environment-only model explained 52.1% of the variation. The full model explained a similar amount of variation (52.6%), and although distance contributed to the fit of the model, it was not a very important predictor, and only increased the percent variation explained by only 0.5% (Figures 1.4d and 1.7b). Neither the Carpentarian Barrier nor the eastern contact zone was retained as an important predictor in the full model.

Discussion

Morphometric variation

As predicted, most morphometric traits were strongly correlated with environmental variables, suggesting a role for ecological selection in shaping their variation. The independent effect of geographic distance on variation in morphometric traits was only detected for wing length, and in this case the effect was minimal. Furthermore, the boundary between genetic lineages (the Carpentarian Barrier) was only selected as an important variable explaining variation in wing length, and again the effect was minimal. These results suggest that morphometric traits are not clearly divergent between the subspecies, but rather that geographic variation in these traits likely arises via local selective pressures from the physical environment, regardless of genetic background. This interpretation is further supported by close examination of the predicted spatial patterns of phenotypic variation. These traits are predicted to vary on a fine geographic scale in

accordance with environmental dissimilarity across the landscape, and the resulting spatial patterns are quite different from what would be expected if one subspecies exhibited clear morphometric differentiation from the other. For example, variation in wing length follows a latitudinal gradient such that birds at similar latitudes appear to have similar wing lengths regardless of whether they are east or west of the genetic boundary between subspecies (Figure 1.4a).

GDM results describe how dissimilarity in phenotypic traits is associated with environmental dissimilarity but they do not provide information about the directionality of these relationships. However, examining the linear relationships between certain morphometric traits and important environmental variables revealed two interesting patterns. First, there was a strong negative relationship between weight and mean temperature (Figure 1.5a). This result suggests that red-backed fairy-wrens may conform to Bergmann's Rule, which posits that organisms will evolve to be smaller in hotter climates to enhance thermoregulation via a more favorable surface area to volume ratio (Bergmann 1847; Mayr 1956). Second, there was a strong positive relationship between tarsus length and percent tree cover (Figure 1.5b), and longer tarsi may be an adaptation enabling the red-backed fairy-wren to better maneuver and forage in trees (*sensu* Grant 1965). I stress that these relationships, although intriguing, are subject to multiple interpretations, and it is difficult to infer evolutionary mechanisms from correlations at such a broad scale. For example, in the case of tarsus length, greater tree cover may influence other aspects of the microhabitat (e.g., understory composition) not included in this study that may affect the evolution of tarsus length more directly.

Interestingly, the association between environment and bill morphology was much weaker than that for other (non-bill) morphometric traits, suggesting a relative lack of fit between bill morphology and environment. This pattern may be explained by the foraging ecology of the red-backed fairy-wren, as this species is a generalist insectivore capable of gleaning prey from leaves, foraging on the ground, and extracting prey from spider webs among other strategies (Rowley and Russell 1997, D. Baldassarre, pers. obs.). Ecological selection on

bill morphology may be relatively weak for a generalist of this sort, particularly if a single bill morphology is sufficient to exploit a diversity of prey. This interpretation is consistent with a recent analysis indicating a lack of ecological speciation among insectivorous warblers compared to granivorous finches (Rundell and Price 2009).

Plumage hue variation

In stark contrast to the patterns observed in morphometric traits, I found several lines of evidence supporting a diminished role of environment and a greater role of isolation by distance in explaining variation in plumage hue. First, the distance-only model for plumage hue explained much more variation in the data than did the distance-only models for morphometric traits. Second, when examining the full model for plumage hue, the independent effect of distance was evident, as it was by far the most important predictor of variation in plumage hue (Figure 1.7a). This pattern was maintained when the western-most sampling site was removed from the model (results not shown), indicating that the effect of geographic distance was not an artifact of a disproportionately distant sampling site. Finally, the predicted pattern of spatial variation in plumage hue (Figure 1.4c) was different from that of any morphometric trait: it did not vary in accordance with any of the environmental variables across the species range, but rather exhibited a clear change from one end of the species range to the other, with the greatest turnover occurring across the eastern contact zone. Taken together, these results indicate that isolation by distance is the most likely explanation for variation in plumage hue. Such a pattern is predicted by the hypothesis of divergent sexual selection via a Fisherian mechanism, coupled with genetic drift in signals and responses to those signals during the period when the two subspecies were geographically isolated (Lande 1981). Furthermore, isolation by distance is a particularly plausible mechanism of divergence in the red-backed fairy-wren, as they have extremely limited natal dispersal (Varian-Ramos and Webster 2012).

The finding that environmental variables have relatively little effect on geographic variation in plumage hue has several implications for the evolution and function of this sexual

signal. First, it suggests that the observed divergence in the carotenoid-based plumage color that characterizes the subspecies is likely not a result of differential availability of carotenoids in the environment. If this were the case, I would expect to observe a pattern where red and orange birds occur in environmentally distinct habitats where there might be a different amount or composition of insect prey (these exclusively insectivorous birds likely acquire carotenoids from insect prey, Rowley and Russell 1997). Although I have no data on insect prey diversity or abundances, and do not know which specific environmental variables influence carotenoid abundance, similar environmental data sources and modeling techniques have accurately characterized the distribution of at least two Australian insect species (Bell et al. 2007). Moreover, although availability of carotenoids in the environment can affect plumage color in some species of birds (Hill 1992), in many species, variation in carotenoid-based plumage color is not thought to be due to differential carotenoid intake (Test 1969; Brush 1970), and a recent review concluded that carotenoid-based signals are no more likely to be environmentally influenced than melanin-based signals (Griffith et al. 2006). Similarly, availability of carotenoids does not appear to be responsible for variation in sexual signals in at least some other taxa (e.g., Steffen et al. 2010). Geographic variation in a carotenoid-based plumage signal might be affected by other mechanisms such as a difference in the timing of molt or usage of food resources between subspecies. However, there is no evidence of any such differences, and importantly, such mechanisms would also be imposed by variation in the physical environment, and the resulting association between environment and plumage color would have likely been detected GDM, as was the case for morphometric traits. Thus, currently the most likely explanation is that geographic variation in the plumage signal is conferred by genetically based differences in the physiological ability to extract (from food), absorb, modify, transport, and deposit ingested carotenoids (Brush 1990; McGraw et al. 2003), though other explanations are possible. The most direct way to test this idea would be to directly modify the carotenoid content in the diet of captive birds from different geographic regions (Hill 1992).

Second, these results also suggest that plumage color has not evolved through a sensory

drive mechanism whereby a certain signal transmits more effectively in a particular physical environment (*sensu* Boughman 2002). However, it is possible that the environmental variables included here may not be relevant to color transmission. Analysis on a finer spatial scale with different environmental characteristics (e.g., ambient light) might reveal differences in microhabitat not detected in this study.

Isolation by distance rather than ecological selection seems to have been influential in shaping variation in plumage hue, but the best-fitting model also included the eastern contact zone as a predictor variable, indicating that there is a high degree of dissimilarity in plumage hue across this region. This area, however, is located approximately 300 km east of the boundary between genetic lineages, the Carpentarian Barrier, across which I detected no significant difference in plumage hue. I suggest that the most likely explanation for this mismatch between the genetic boundary and the phenotypic contact zone is the asymmetrical introgression of red plumage from the western *M. m. cruentatus* subspecies into the genetic background of the eastern *M. m. melanocephalus* subspecies. Mathematical models supports the idea that hybridization can facilitate the introgression of an advantageous trait into another population (Anderson and Stebbins 1954; Arnold 1992; Barton 2001). In this case, introgression of red plumage may be driven by sexual selection if red-backed males have a sexually selected advantage via male competition (Pearson and Rohwer 2000) or female choice (Stein and Uy 2006). Finer-scaled spatial analyses of the possible lack of coincidence between genetic and phenotypic clines as well as field experiments are the subjects of Chapters 2-4.

Male tail length variation

Male tail length is presumed to be an intrasexual signal used in male competition (Karubian et al. 2009), and I predicted that it would exhibit a pattern of variation similar to plumage hue. Instead, the pattern of variation in male tail length was more similar to those of morphometric traits, where environmental variables were retained as important predictors of variation, but geographic distance was not. In fact, the predicted pattern of spatial variation in male tail length was very

similar to that of wing length (both are predicted to follow a latitudinal gradient, compare Figures 1.4a and d). Geographic distance explained more variation in male tail length than in the morphometric traits, but this effect was not as strong as in the models of plumage hue. This pattern suggests that evolution of male tail length may be constrained by ecological selection if tail length impacts survival in some way. Red-backed fairy-wrens are not strong fliers and maneuver through their habitat primarily by hopping and making short flights (Rowley and Russell 1997, D. Baldassarre, pers. obs.), so the tail may play an important role in stability and balance during locomotion. This constraint may explain why there is not a clear divergent pattern of isolation by distance as is seen in plumage hue, despite a trend for *M. m. cruentatus* males to have shorter tails than *M. m. melanocephalus* males (Rowley and Russell 1997).

Conclusions

In summary, comparing the influence of environment on sexual and non-sexual traits suggests that different evolutionary forces have shaped geographic variation in these traits. The red-backed fairy-wren exhibits variation in a number of morphometric traits such as weight and wing length that is well explained by environmental variation but does not show a clear pattern of divergence between the subspecies. These results suggest that ecological selection has acted on these traits to create geographic variation that is independent of subspecies identity, such that individuals exhibit morphometric traits that are adapted to the local environment. In contrast, variation in plumage hue, the most salient difference between the subspecies, is not well explained by environment, but rather shows a strong pattern of isolation by distance across the subspecies, with a particularly high rate of plumage hue change across the eastern contact zone. This pattern suggests that geographic variation in hue likely has evolved via a Fisherian mechanism, beginning with a period of stochastic divergence (Lande 1981; Uyeda et al. 2009). Upon secondary contact, red plumage appears to have introgressed across the genetic boundary between the subspecies, possibly driven by sexual selection. The evolutionary forces acting on male tail length appear to be more complex, as variation in this putative signal seems to be

constrained to some extent by the environment, and male tail length has likely not evolved purely via stochastic or sexually selected processes. This study highlights the importance of considering sexual selection in combination with stochastic processes when examining the evolution of sexual signals, including those predicted to be under strong environmental influence. In addition, there is evidence that two putative sexual signals have evolved via different evolutionary pathways, with variable selective pressures from the physical and social environments. Further experimental studies are needed to test the information content and function of male plumage color and tail length in multiple populations.

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Table 1.1 The 21 predictor variables included in this study. I considered the influence of 18 environmental variables, geographic distance, the Carpentarian Barrier, and the eastern contact zone on phenotypic variation. Variable numbers are referred to in Table 1.2.

Variable number	Data source	Variable name	Variable interpretation
1	WorldClim database	Bio1	Mean temperature
2		Bio2	Diurnal temperature range
3		Bio4	Temperature seasonality
4		Bio5	Maximum temperature of warmest month
5		Bio6	Minimum temperature of coldest month
6		Bio12	Annual precipitation
7		Bio15	Precipitation seasonality
8		Bio16	Wet season precipitation
9		Bio17	Dry season precipitation
10		Bio18	Precipitation of warmest quarter
11		Bio19	Precipitation of coldest quarter
12	MODIS satellite spectroradiometer	NDVImax	Maximum vegetation production
13		NDVImean	Mean vegetation production
14		NDVIsd	Vegetation seasonality
15	QuickScat active radar scatterometer	Qscatmean	Mean surface moisture
16		Qscatsd	Surface moisture seasonality
17	Shuttle Radar Topography Mission	Elevation	
18	MODIS satellite spectroradiometer	Percent tree cover	
19	GPS	Geographic distance	
20		Carpentarian Barrier	
21		Eastern contact zone	

Table 1.2 Results of each generalized dissimilarity model for all phenotypic traits. For each trait, four separate models were run. Reported for each model are the percent of variation in the data explained, and the predictor variables that were retained in the model. Full models include isolating barriers if they were retained as important predictors. In parentheses are the most important predictor variables (with the highest response curve or response curve heights ≥ 50% of the highest), listed in order of relative importance. The remaining predictor variables are listed in numerical order. Variable numbers are taken from Table 1.1.

	Full model		Environment-only		Distance-only	Random variables
Trait	% var. explained	Predictor variables	% var. explained	Predictor variables	% var. explained	% var. explained
Wing	31	(5) 2,6,7,10,16,17,18,20	30.3	(5) 3,6,9,13,16,17,18	0.9	4.1
Tarsus	46.5	(17,14,18,15) 4,6,7,10,11,16	46.5	(17,14,18,15) 4,6,7,10,11,16	2.1	2
Tail	31.4	(3) 2,5,6,7,8,9,13,16,18	31.4	(3) 2,5,6,7,8,9,13,16,18	11.5	3.9
Weight	56.5	(7,1) 9,11,14,15,16,17	56.5	(7,1) 9,11,14,15,16,17	0.9	9.9
Bill + Head	38.9	(16,18) 2,3,5,7,10,11,14,15,17	38.9	(16,18) 2,3,5,7,10,11,14,15,17	6.4	0.7
Culmen	11.7	(7,11) 3,10,15,17	11.7	(7,11) 3,10,15,17	1	7.2
Bill Depth	28.6	(7) 1,4,11,12,13,14,15,17	28.6	(7) 1,4,11,12,13,14,15,17	2.4	1
Bill Width	13.4	(2,15,17,11) 3,7,18,21	12.9	(2,15,17) 3,7,11	2.4	3
Male Tail	52.6	(5,10,3) 7,8,9,15,19	52.1	(5,10,3) 1,7,8,9,15,16	13.5	4.1
Plumage Hue	78.9	(19) 2,3,6,7,9,11,15,21	62.6	(7) 1,2,4,9,11,12,15,16,17,18	47.6	10.4

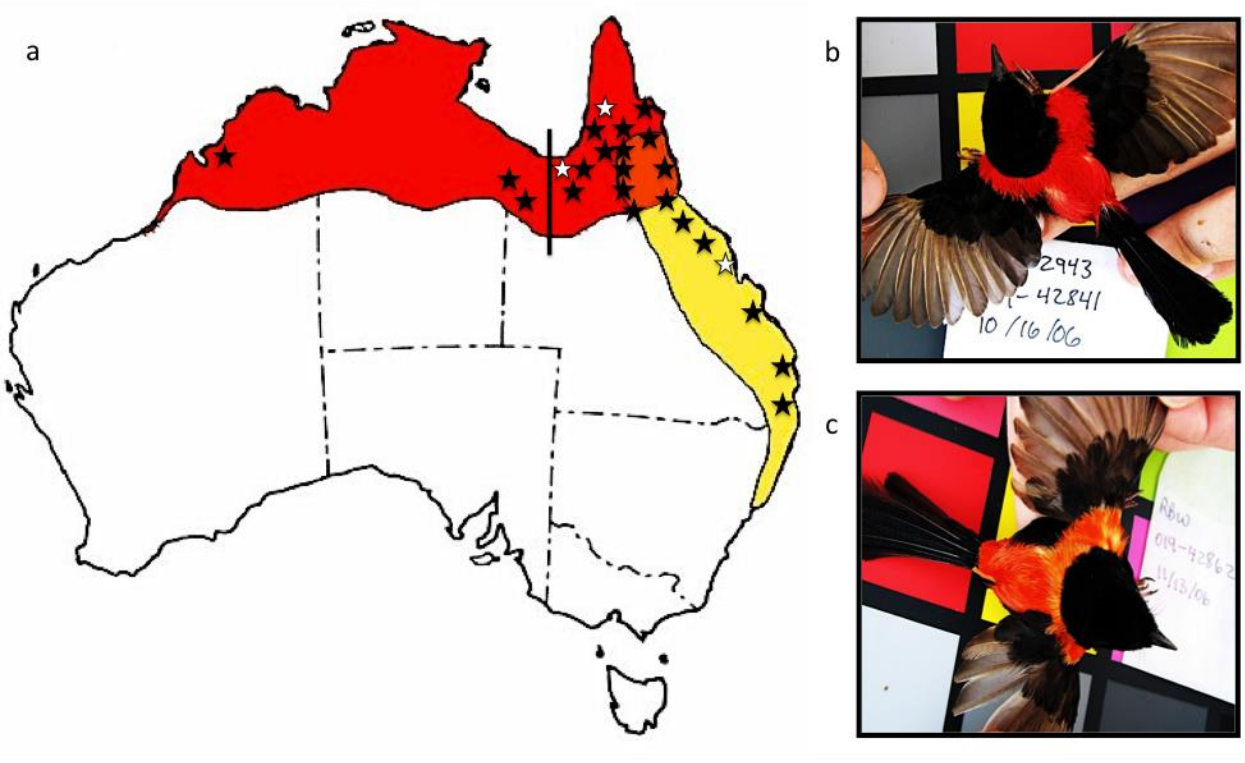

Figure 1.1 The species range of the red-backed fairy-wren. The species occurs across northern Australia, in the Cape York Peninsula, and along much of the east coast (a). The range crimson-backed, shorter-tailed *M. m. cruentatus* subspecies is distributed in the west and north (b); and the orange-backed, longer-tailed *M. m. melanocephalus* subspecies is distributed along the east coast (c). Field observations have led to the subjective delineation of a morphological contact zone in the northeast. The solid line represents the Carpentarian Barrier, a biogeographic barrier across which the subspecies are genetically differentiated. Stars indicate sampling localities (N = 24) and white stars indicate three locations not included in the plumage color and male tail length dataset.

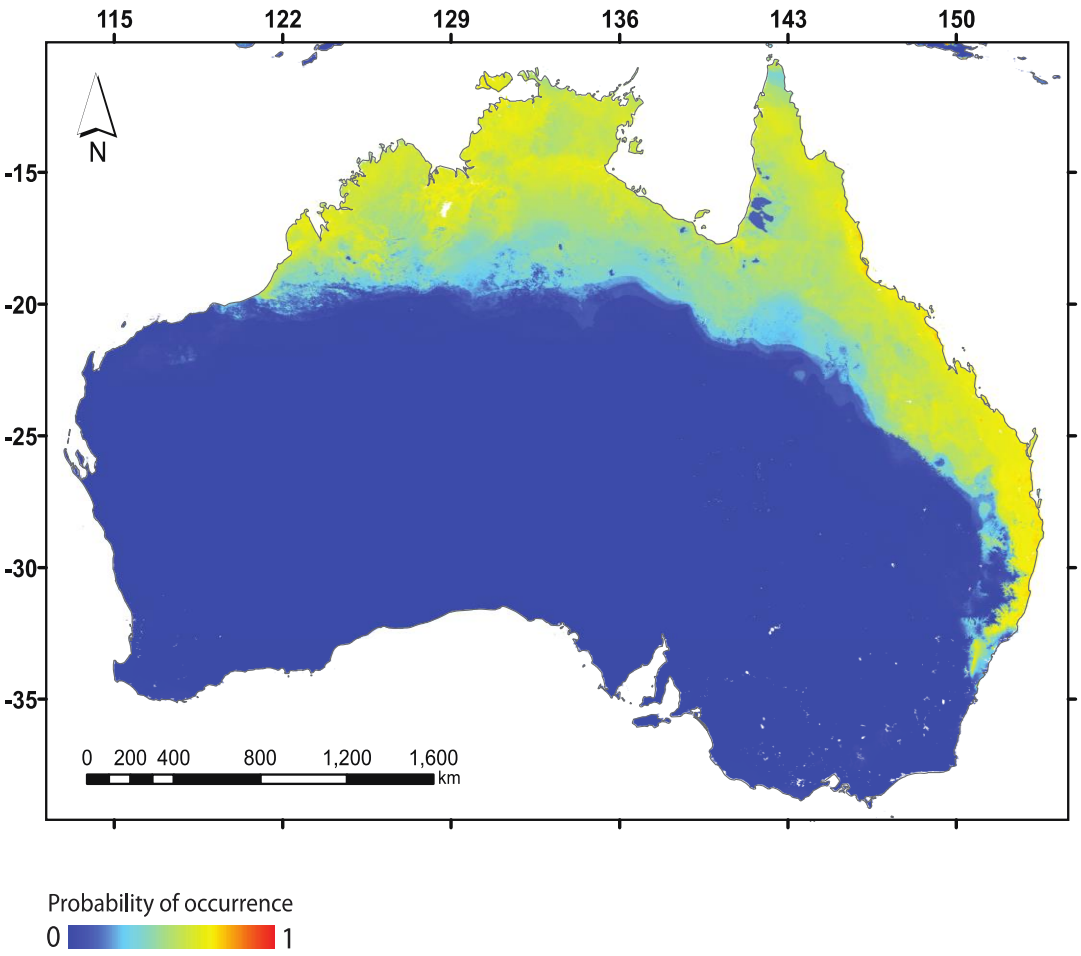

Figure 1.2 Distribution of the red-backed fairy-wren as modeled by Maxent 3.0. Warmer colors indicate more suitable environmental conditions; cooler colors indicate less suitable conditions. See text for details on model parameters.

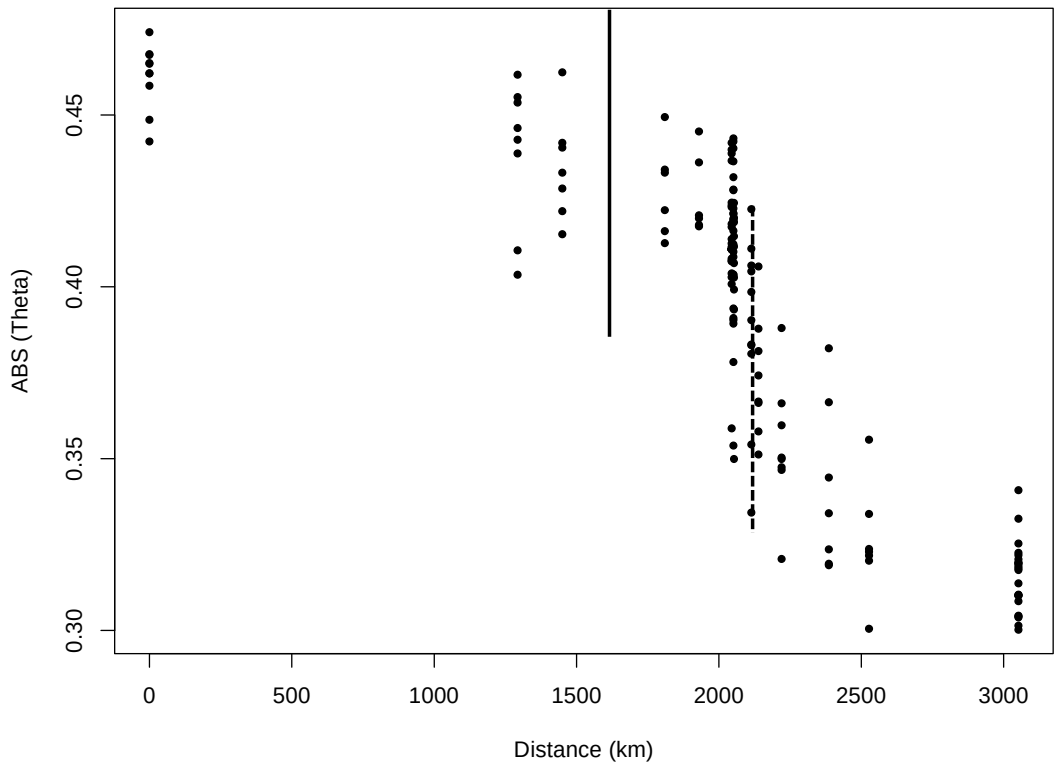

Figure 1.3 Plumage hue across the red-backed fairy-wren species range. The western-most sampling location (Mornington) was assigned distance 0, and distance 3,052 km represents the sampling location in the far southeast of the species range. Distances were calculated as the shortest straight-line distance between a given location and Mornington except in cases where this path would cross into unsuitable habitat as determined by the species distribution model (see Figure 1.2). In these cases, the distance was calculated as the straight-line distance from Mornington to a "pivot point" on inner edge of the range, plus the straight-line distance from the "pivot point" to the given location. Values for theta (hue) were converted to absolute values for visualization purposes, with larger values indicating redder hue. The result is a cline describing a transition from redder plumage in the west to more orange plumage in the southeast. The approximate center of the cline was computed as the distance along the cline that encompassed the median theta value (dashed line). This region was used as the eastern contact zone in GDM analyses. The solid line represents the genetic boundary between subspecies, the Carpentarian Barrier.

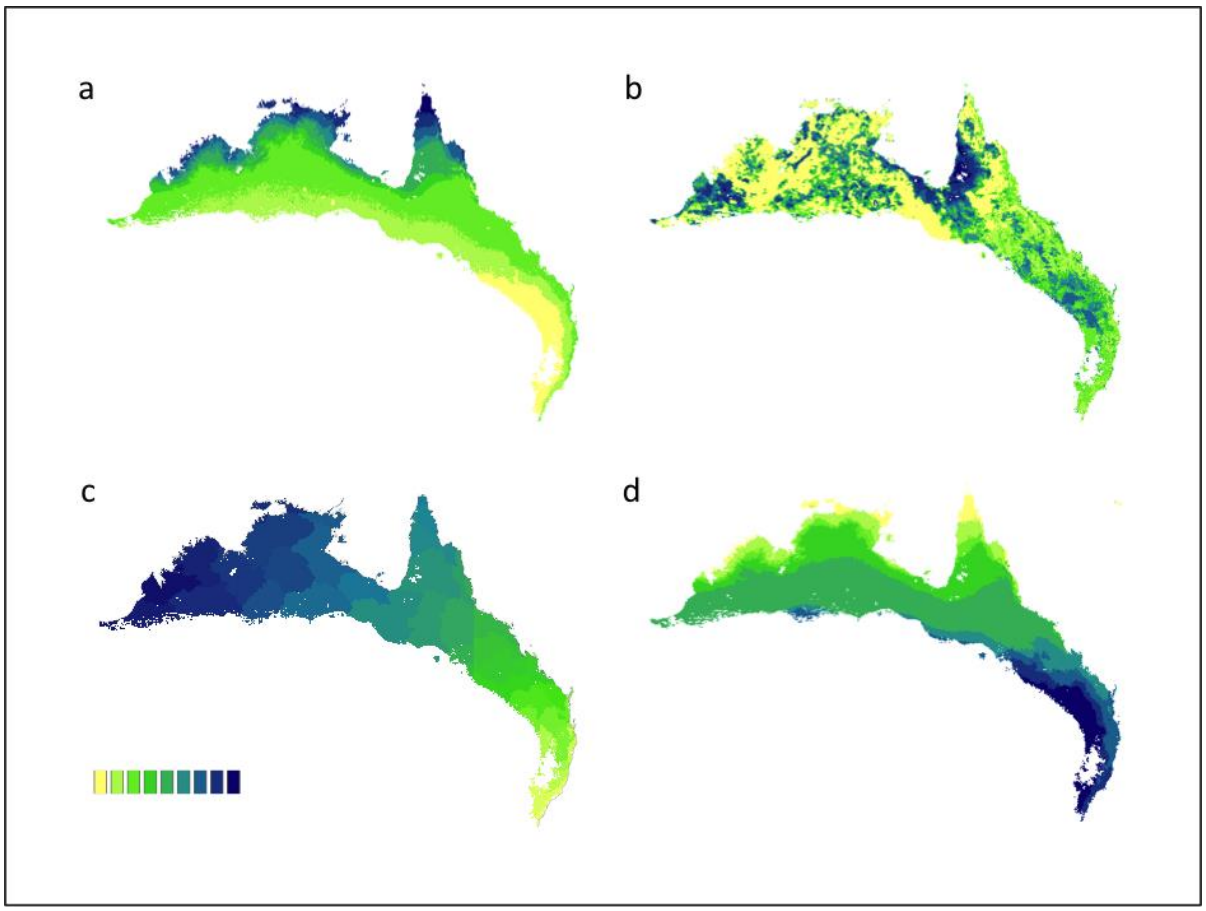

Figure 1.4 Predicted spatial patterns of phenotypic variation. Shown are model results for wing length (a), bill plus head length (b), plumage hue (c), and male tail length (d), as determined by the full generalized dissimilarity model for each trait. Maps for the remaining morphometric traits are not shown. Differences in color are proportional to differences in the trait value across the landscape (see color bar for scale).

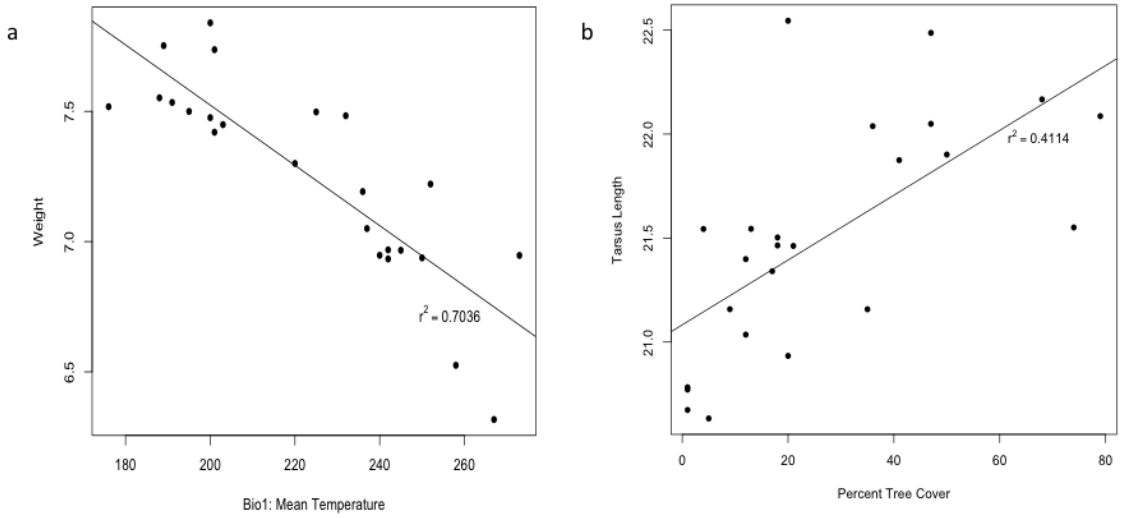

Figure 1.5 Relationships between environmental variables and two morphometric traits. Linear regressions of weight (g) on Bio1: mean temperature (a), and tarsus length (mm) on percent tree cover (b). Both linear regressions are significant at $p < 0.001$.

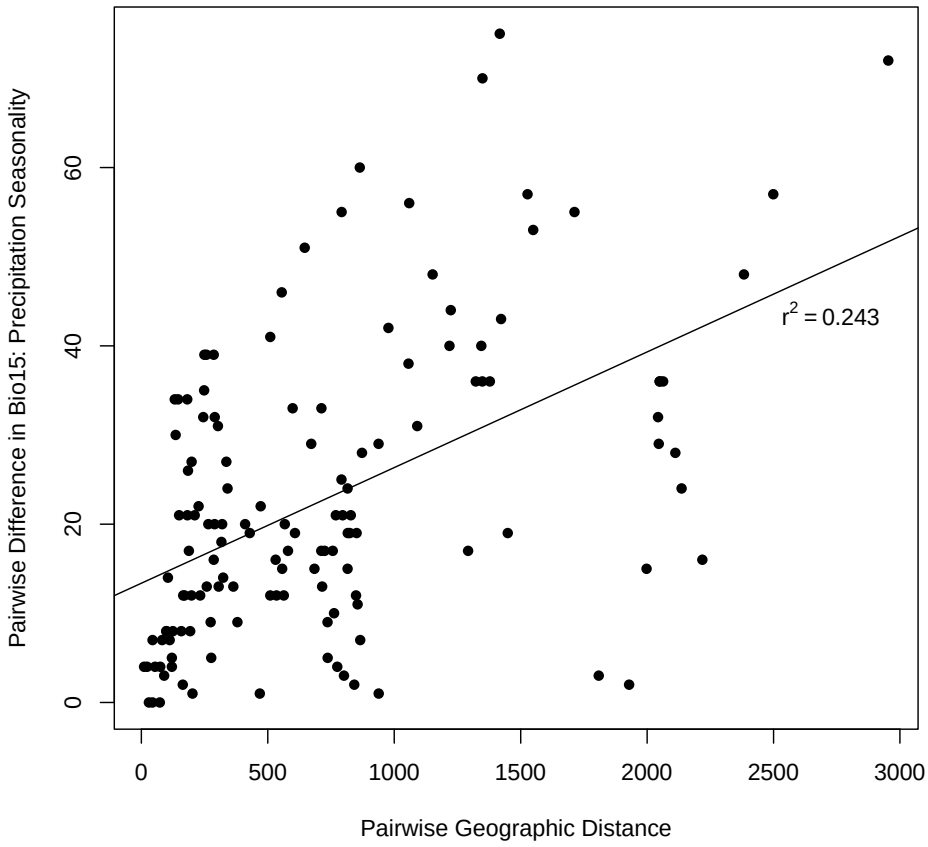

Figure 1.6 The spatial correlation between geographic distance and environmental dissimilarity across the red-backed fairy-wren species range. A linear regression of pairwise difference in Bio15: precipitation seasonality on geographic distance among sites in the RBFW species range is highly significant ($p < 0.001$).

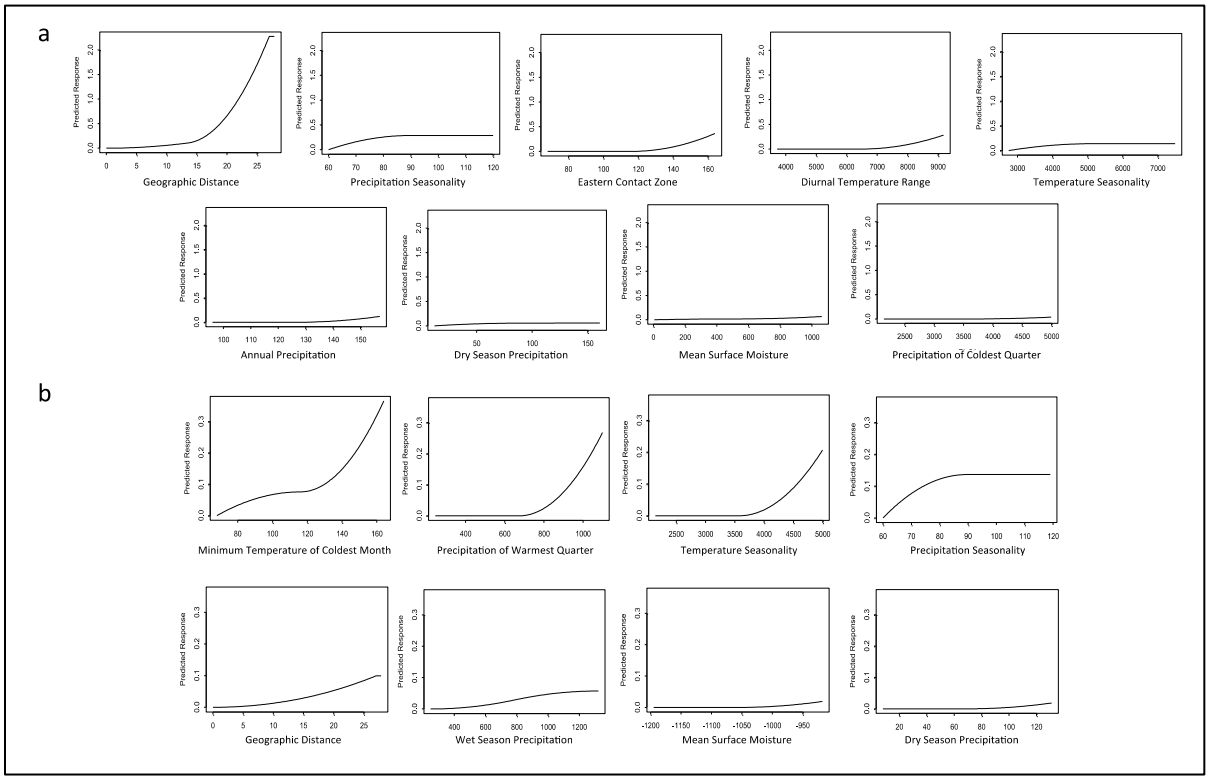

Figure 1.7 Important predictor variables describing variation in sexual signals. Panels show response curves of input variables retained as significant predictors of variation in the full model of plumage hue (a) and male tail length (b). Maximum height is indicative of the relative importance of each input variable, and the slope indicates the predicted rate of change in the response variable as a function of the predictor variable. Response curves for models of morphometric traits are not shown.

CHAPTER 2

GENOMIC AND MORPHOLOGICAL ANALYSIS OF THE RED-BACKED FAIRY-WREN HYBRID ZONE REVEALS ASYMMETRICAL INTROGRESSION OF A SEXUAL SIGNAL

Abstract

Hybrid zones are geographic regions where taxa with differentiated genomes meet and potentially exchange genes. Increasingly, detailed genomic analyses have demonstrated that many hybrid zones are semi-permeable boundaries across which introgression is highly variable. In some cases, alleles from one parental taxon penetrate across the hybrid zone in one direction, recombining into the other genome. I investigated this phenomenon using genomic (genotyping-by-sequencing) and morphological (reflectance spectrophotometry of plumage) analyses of the hybrid zone between two subspecies of the red-backed fairy-wren (*Malurus melanocephalus*) that differ conspicuously in a sexual signal, male back plumage color. Geographic cline analyses revealed a highly variable pattern of differential introgression, with many narrow coincident clines combined with several significantly wider clines, suggesting that the hybrid zone is a semi-permeable tension zone. The plumage cline was shifted significantly into the genomic background of the orange subspecies, suggestive of sexual selection driving asymmetrical introgression of red plumage across the hybrid zone, potentially compounded by genetic dominance. This interpretation is supported by experimental work in Chapter 3 demonstrating an extra-pair mating advantage for red males. This study highlights the potential for sexual selection to erode species boundaries and promote gene flow, particularly when taxa are at an intermediate stage of divergence.

Introduction

Hybrid zones are geographic regions where two genetically differentiated taxa distinguished by at least one heritable trait meet and interbreed, thereby potentially exchanging genes (Harrison 1990). Theory suggests that many hybrid zones are likely semi-permeable boundaries between genomes, where some genes are free to introgress, whereas others are not, depending on their effects on fitness and reproductive isolation, as well as their genetic linkage relationships (Barton 1979b; Harrison 1986; 1990; Wu 2001; Morjan and Rieseberg 2004; Gompert et al. 2013). This idea is gaining empirical support with the advent of genomic tools (Nosil et al. 2007; Fitzpatrick et al. 2009; Gompert et al. 2010; Larson et al. 2014). In these cases, genes that contribute to local adaptation or reproductive isolation can cause regions of the genome to remain differentiated even in the face of ongoing gene flow at other loci. Alternatively, if the cost of hybridization is low, gene flow at certain loci may be relatively unrestricted and even beneficial (i.e., adaptive introgression, Anderson and Stebbins 1954; Arnold 2004; Hedrick 2013). Semi-permeability may be particularly likely in hybrid zones between taxa at an intermediate stage of divergence where some barriers to gene flow have evolved, but reproductive isolation is incomplete. Asymmetrical introgression is a special case of differential gene flow that can occur within such hybrid zones, where particular alleles from one parental genome flow in one direction but not the other, thereby penetrating into the alternative genetic background.

Many cases of asymmetrical introgression of neutral markers have been documented (reviewed in Harrison 1990), but asymmetrical introgression of genes underlying adaptive traits from one taxon into the other appears to be much less common (but see Parsons et al. 1993; Brumfield et al. 2001; Dasmahapatra et al. 2012; Pardo-Diaz et al. 2012). Mathematical models suggest that strong positive selection in the alternate genetic background and/or environment is necessary for asymmetrical introgression to occur (Barton 1979b; 2001; Hedrick 2013). Several cases of asymmetrical introgression involve secondary sexual characteristics and appear to be driven by asymmetrical mating preferences (e.g., Stein and Uy 2006; Garner and Neff 2013), which are predicted to be most prevalent when taxa are at an intermediate stage of divergence

(Arnold et al. 1996). In birds, recent theory suggests that extra-pair mating may be a potent mechanism promoting hybridization and introgression if females prefer heterospecific males as extra-pair mates (Hartman et al. 2011). Thus, there is mounting evidence that sexual selection, particularly among sexually promiscuous species, can erode species boundaries and promote introgression. This is in stark contrast to the large body of theory (Lande 1981; West-Eberhard 1983; Uyeda et al. 2009) and empirical research (reviewed in Panhuis et al. 2001; Ritchie 2007) on the capacity for sexual selection to promote population divergence and speciation.

The red-backed fairy-wren (*Malurus melanocephalus*), a small insectivorous passerine bird endemic to Australia, appears to exhibit asymmetrical introgression of a sexual trait. This species is highly sexually dichromatic (Schodde 1982; Rowley and Russell 1997) and exhibits very high rates of extra-pair paternity (Karubian 2002; Webster et al. 2008; Varian-Ramos and Webster 2012; Varian-Ramos et al. 2012; Baldassarre and Webster 2013) and large testes size (Rowe and Pruett-Jones 2011; 2013), suggesting that males are subject to strong sexual selection. Moreover, two distinct subspecies are recognized based on variation in a sexual signal, male nuptial plumage color: the western, red-backed *M. m. cruentatus* and the eastern, orange-backed *M. m. melanocephalus* (Schodde 1982; Rowley and Russell 1997, Figure 2.1). Across populations, there is considerable variation in other non-sexual traits, but variation in these traits is spatially complex, and is not broadly diagnostic of the two subspecies (Chapter 1; Baldassarre et al. 2013). The two subspecies are moderately genetically differentiated across the Carpentarian barrier (Lee and Edwards 2008), a prominent zoogeographic barrier in northwestern Queensland that likely forced subspecies into coastal refugia during the Pleistocene (Cracraft 1986; Chivas et al. 2001; Bowman et al. 2010; Cook et al. 2012). Secondary contact between the subspecies likely occurred once the climate ameliorated (Lee and Edwards 2008). A contact zone also exists between plumage types, but is located east of the Carpentarian Barrier and does not appear to be coincident with the location of secondary contact (Chapter 1; Baldassarre et al. 2013, Figure 2.1). Previously, I suggested that this lack of coincidence was due to asymmetrical introgression of red back plumage color across the hybrid zone, likely facilitated by sexual selection via extra-

pair mating (Chapter 1; Baldassarre et al. 2013; Baldassarre and Webster 2013).

Despite these multiple lines of evidence, a thorough genetic and morphological analysis of the hybrid zone has not been conducted to rigorously test the hypothesis that red plumage color has introgressed asymmetrically. Geographic cline theory provides a suitable framework for testing these types of hypotheses by analyzing changes in allele frequencies and quantitative traits as a function of geographic distance along a transect through a hybrid zone (Barton and Hewitt 1985; Szymura and Barton 1986). Alleles and traits that are subject to similar selective pressures are predicted to have similar cline centers and widths, whereas those subject to positive selection on one side of the hybrid zone will have cline centers that are displaced from the majority of other clines (Barton 1983; Barton and Hewitt 1985). Using the reduced representation genomic approach genotyping-by-sequencing (GBS), I developed a large, genome-wide, single nucleotide polymorphism (SNP) dataset that I compared in a clinal framework to variation in plumage color across a 3,052 km transect through the hybrid zone. The major goals of my analyses were to employ dense geographic sampling and high resolution genomic data to test the hypotheses that 1) the hybrid zone as determined by genetic markers is centered at the Carpentarian Barrier (*sensu* Lee and Edwards 2008), and 2) alleles for red plumage color have introgressed asymmetrically from *cruentatus* into the genetic background of the orange *melanocephalus* subspecies. I predicted that the majority of genetic clines would have similar centers and widths located near the Carpentarian Barrier, whereas the plumage color cline would have a center shifted significantly into the background of the orange species. Although I did not make any *a priori* predictions about patterns of genetic introgression among SNP loci, I used geographic cline theory to explore the extent of locus-specific introgression relative to dispersal, to make inferences about the strength of selection in the hybrid zone. I also consider the possibility that non-selective mechanisms (e.g., dominance, epigenetic effects) may explain the apparent lack of coincidence between the hybrid zone and the plumage contact zone.

Methods

Field methods and sampling transect

From 2004 through 2011, colleagues and I collected blood samples for genetic analyses and feather samples for plumage color analyses at sampling locations across the range of the red-backed fairy-wren (Figure 2.1). The genetic dataset consisted of 169 individuals from thirteen sampling locations, and the plumage color dataset consisted of 178 individuals from fifteen sampling locations (Table 2.1). Birds were captured using mist nets and a small (ca. 50 μl) blood sample was collected and immediately stored in lysis buffer for subsequent DNA extraction. Feather samples were collected by removing a small number (ca. 6-12) of feathers from the center back of every captured male in nuptial plumage. I chose to collect feather samples instead of voucher specimens because long-term behavioral studies were being conducted at many of the sampling locations. For geographic cline analyses, I arranged sampling locations along a transect beginning with the western-most location (Mornington), and ending at the location in the far southeast of the range (Brisbane). Each sampling location was assigned a distance along the transect that corresponded to the shortest straight-line distance between it and Mornington, calculated using Google Earth. In certain cases, this path would cross into unsuitable habitat where red-backed fairy-wrens do not occur. In these instances, I created a "pivot point" on the interior edge of the species range, and calculated the distance as the sum of the two straight-line distances passing through this point (Chapter 1; Greig and Webster 2013; Baldassarre et al. 2013). This resulted in a 3,052 km transect spanning very nearly the entire species range.

Plumage color quantification

I quantified plumage color following the methods of Chapter 1 and Baldassarre et al. (2013). From each male's feather sample, we mounted six feathers in an overlapping pattern on a square of black construction paper (Strathmore Artagain® Coal Black). I used an Ocean Optics USB2000 UV-VIS spectrometer with a R200-7 UV-VIS probe and a PX2 pulsed xenon light source to measure the reflectance of the feather sample. The probe tip was adjusted to illuminate a measurement area of 3 mm^2, and was mounted in a metal block to exclude ambient light. I

measured three separate reflectance curves along the avian visible spectrum (300 nm-700 nm) for each feather sample, and averaged the three curves for further analyses.

To account for the unique tuning of the avian visual system, I analyzed reflectance curves using the program TetraColorSpace (Stoddard and Prum 2008), which uses the spectral sensitivity of each of the four cone types in the avian retina to plot each color as a point in a tetrahedral color space. The red-backed fairy-wren has a violet-sensitive visual system (Odeen et al. 2012), so I analyzed curves using the average avian violet-sensitive spectral sensitivity curve (Endler and Mielke 2005) assuming idealized illumination (i.e., I did not consider variation in ambient light). TetraColorSpace produces several values that quantify the hue, saturation, and brightness of a color. For this study, I focused on one measure of hue, theta (θ), because previous analyses have shown that it best captures the variation in red to orange that characterizes the subspecies (Chapter 1; Baldassarre et al. 2013; Baldassarre and Webster 2013).

Genotyping-by-sequencing

DNA was extracted from blood samples using the Omega Bio-Tek EZ 96 Total DNA/RNA Isolation Kit®, eluted in water, and then concentrated using a vacuum centrifuge. GBS libraries were prepared and analyzed at the Institute for Genomic Diversity (IGD) at Cornell University (Elshire et al. 2011), using the enzyme PstI for digestion. GBS libraries were sequenced on two lanes of an Illumina HiSeq 2000 (100 base pair (bp), single-end, 96 unique barcodes per lane) at the Cornell University Life Sciences Core Center. The resulting raw Illumina data files were filtered into individual SNP genotypes using the non-reference genome Universal Network Enabled Analysis Kit (UNEAK) pipeline (Lu et al. 2013), which is implemented in TASSEL 3.0 (Bradbury et al. 2007). UNEAK trims all reads to 64 bp after the barcode sequence, clusters identical reads into tags, and then retains the counts of tags in each barcoded individual. The pipeline then performs a pairwise alignment of all tags, and tag pairs with one bp mismatches are considered candidate SNPs. Reciprocal pairs of tags are considered true SNPs according to standard protocols at the IGD, with a error tolerance rate of 0.03. After identifying true SNPs,

UNEAK returns the count of alleles for each locus in each individual.

Following UNEAK filtering, individual genotypes were re-called using the methods described in (Lynch 2009; White et al. 2013). Genotype likelihood was calculated using a binomial distribution, and a given genotype was called if its AIC value was at least four lower than the next best genotype, otherwise it was considered missing. I filtered out potential paralogs by discarding loci with a mean heterozygosity > 0.75. Although this cutoff is somewhat arbitrary, varying the mean heterozygosity threshold between 0.5 and 1 had no major effect on the results (data not shown). Lastly, I generated two datasets for which either 20% or 40% of the individuals did not have sequence data at a known SNP locus (i.e., missing data).

I sequenced 190 individuals on two lanes of the Illumina HiSeq platform, generating 833,545,374 reads. 169 individuals passed initial filtering and were run through the UNEAK pipeline, resulting in 11,136 biallelic SNP loci. Finally, excluding loci with 20% or 40% missing data resulted in 645 loci with a mean coverage of 19x and 2,702 loci with a mean coverage of 12x respectively. Results for datasets with different amounts of missing data were similar (data not shown), so I present here only the results for the larger dataset that allowed 40% missing data.

Population structure analyses

To explore the genetic structure of the hybrid zone and develop a genomic hybrid index, I analyzed all 2,702 SNP loci and a subset of 102 "diagnostic" loci (one allele had a frequency > 0.8 on one end of the cline and < 0.2 on the other end) with a Bayesian clustering method implemented in the program STRUCTURE v. 2.3.4 (Pritchard et al. 2000; Falush et al. 2003). Without using *a priori* information about sampling locations, STRUCTURE assigns individuals to genetic groups minimizing Hardy-Weinberg disequilibrium. Because previous phylogeographic analyses suggested the presence of two separate lineages (Lee and Edwards 2008), and because I were primarily interested in investigating the dynamics of the hybrid zone, we set the number of genetic clusters (K) = 2. However, increasing K did not provide a better

explanation of the data as measured by log likelihood (data not shown). I ran ten iterations with $K = 2$, using the admixture model with correlated allele frequencies, a burn-in period of 100,000, and 100,000 Markov chain Monte Carlo replicates, after which all parameters converged. I used the probability that an individual was assigned to the western *cruentatus* cluster considering its multilocus genotype by STRUCTURE (Q), as the hybrid index (Singhal and Moritz 2013). I generated two hybrid indices: one using the inclusive 2,702 loci dataset (hereafter HI), and one using the 102 "diagnostic" locus dataset (hereafter HI_{diag}). I then used an individual's hybrid index score to assign it to a hybrid category (i.e., pure parental or mixed ancestry) using the classification scheme of Aboim et al. (2010) and Taylor et al. (2012). An individual was classified as pure *cruentatus* if $Q \geq 0.9$, pure *melanocephalus* if $Q \leq 0.1$, and mixed ancestry if $0.9 > Q > 0.1$.

Geographic cline analyses

Allele frequencies for each SNP locus, the two hybrid indices (HI and HI_{diag}), and plumage hue were fit to a series of equilibrium geographic cline models (Szymura and Barton 1986; Gay et al. 2008) using the Metropolis-Hastings Markov chain Monte Carlo (MCMC) algorithm employed in the R package *HZAR* (Derryberry et al. 2013). I ran fifteen separate models that varied in the number of cline shape parameters estimated. All models estimated cline center (distance from sampling location 1, c) and width (1/maximum slope, w), but could additionally estimate different combinations of the exponential decay curve (tail) parameters δ and τ (neither tail, right tail only, left tail only, mirrored tails, or both tails separately). Parameters δ and τ are analogous to d and t described by Gay et al. (2008) and represent the distance from the cline center to the tail and the tail slope respectively. In addition, the genetic models varied as to whether they did or did not estimate allele frequencies at the cline ends (p_{min} and p_{max}) or fixed them at 0 and 1. The plumage hue models varied as to whether they did or did not estimate mean and variance at the cline ends. These models were then compared using Akaike information criterion corrected for small sample size (AICc). For each trait, the model with the lowest AICc score was selected

as the best-fitting model.

Sample sizes for the genetic clines were corrected following (Raufaste et al. 2005) as

$$N_e = \frac{2N}{2N * F_{ST} + F_{IS} + 1}$$

where N is the number of individuals sampled in a deme, F_{IS} is the deficit of heterozygotes (zero if not positive), and F_{ST} is the fluctuation of allele frequencies between loci, after accounting for differences in their cline shapes. F_{ST} is calculated from the residual variation around the regression line fit during the concordance analysis (see below). For the hybrid indices, I averaged N_e across all loci at each sampling location.

To determine if an individual SNP cline exhibited a different cline shape from the hybrid index, I analyzed the relationship between the hybrid index and allele frequency at each individual locus using the logit-logistic model of Fitzpatrick (2013). Again, I restricted this analysis to SNP loci with one allele that had a frequency > 0.8 on one end of the cline and < 0.2 on the other end. The predicted allele frequency p in deme i is given by

$$p_i = \frac{S^{v_i}}{S^{v_i} + (1 - S)^{v_i} e^{u_i}}$$

where S is the mean hybrid index over all loci, u gives the relative difference in cline position, and v gives the relative difference in slope. Perfect concordance between a focal locus and the mean hybrid index would result in $u = 0$ and $v = 1$. Parameters u and v were fit using the function mle2 in the R package *bblme*. Two further models were also fit where either u or v was constrained to be 0 or 1, respectively, and these were compared to the unconstrained model using likelihood ratio tests. *P*-values were adjusted using the Bonferroni correction.

I used two approaches to test for coincidence between the center of the plumage cline and the genetic clines. First, I compared the two log-likelihood unit support limits (hereafter CIs) for

the plumage cline center to that of the hybrid index and average SNP clines, and considered them non-coincident if they did not overlap. I also compared the CIs for plumage cline center and each individual SNP locus. Second, I re-fit the best-fitting plumage cline model but fixed the value for *c* at the estimated cline center for the two hybrid index clines. I compared the AICc score of each constrained model with the free model and considered it a worse fit, and thus evidence of non-coincidental clines, if it had an AICc score more than two points higher.

Dispersal and selection in the hybrid zone

To calculate the width of a neutral cline and effective selection on a locus, I first estimated root-mean-square (RMS) dispersal distance from four years of mark-recapture data at my field site near Brisbane, Queensland (sampling location 16, Figure 2.1). Dispersal in this species is sex-biased toward females (Varian-Ramos and Webster 2012), so I only included data from 16 observed female natal dispersal events. Determining the width of a neutral cline also requires estimating the time since secondary contact. Assuming that widespread aridity across the Carpentarian Barrier was the primary barrier separating the subspecies during the Pleistocene, I estimated that the most recent time for secondary contact was 21 kya, when aridity was most severe, and after which time the climate ameliorated rapidly (Bowler 1976; Torgersen et al. 1988; Chivas et al. 2001; Williams et al. 2009). Free introgression of neutral alleles across the hybrid zone will result in wide, symmetrical clines (i.e., neutral diffusion, Endler 1977; Barton and Gale 1993). When this is the case, the width of a cline (w) can be modeled as a result of time since secondary contact (t) and dispersal distance (σ) following equation (Barton and Gale 1993):

$$w = 2.51\sigma\sqrt{t}$$

This cline width can then be compared to the observed cline widths to determine the likelihood that the observed clines are the result of neutral diffusion or, if narrower, the result of a barrier to gene flow preventing further introgression. I considered an observed cline width to be narrower

than the neutral cline if its upper 95% confidence limit did not overlap with the above-calculated cline width. Furthermore, I used the estimate of dispersal distance to calculate the effective selection pressure on a locus following Barton (1979b), relating cline width (w) to dispersal distance (σ) and selection (s):

$$w = \sigma\sqrt{(8/s)}$$

Results

Genomic analysis of hybrid zone

STRUCTURE analyses showed a clear geographic pattern of hybridization that did not differ qualitatively depending on whether all 2,702 or the subset of 102 diagnostic SNP loci were analyzed (Table 2.1, Figure 2.2). The 2,702 loci analysis revealed that all individuals from sampling locations 1-4 had a ≥ 0.9 (mean = 0.99 ± SD 0.01) probability of belonging to the *cruentatus* genetic cluster (i.e., hybrid index, *Q*), and 98% of individuals from sampling locations 7-16 had a *Q* value ≤ 0.1 (mean = 0.01 ± SD 0.023). All individuals from locations 5 and 6 had mixed ancestry, with a mean *Q* value of 0.64 ± SD 0.04 and 0.33 ± SD 0.08, respectively.

Individual SNP clines were highly variable in both center (mean = 1,721.7 ± 305.3 km, range = 736.3-2,989.8 km) and width (mean = 578.9 ± SD 110 km, range = 12.3-3,249.9 km, Figure 2.3, Appendix 2.1). There was considerable variation in the identity of the best-fitting model for each locus, but the most common model was the one where p_{min}/p_{max} were fixed at 0 and 1 and no tail parameters were estimated (62/102 loci). This was also the best-fitting model for both the HI and HI_{diag} clines (Table 2.2). Estimates for center and width of the two hybrid index clines were very similar, with overlapping CIs: HI c = 1,753.2 (1,673.9-1,813) km, w = 251.3 (131.7-451.6) km; HI_{diag} c = 1,730.4 (1,648.5-1,790.7) km, w = 238.5 (114.4-438.8). Corroborating the STRUCTURE results, the average SNP, HI, and HI_{diag} cline centers were all located between sampling locations 5 and 6 (Figure 2.3). Likelihood ratio tests based on the logit-logistic model indicated that 42% (43/102) of SNP clines could not be constrained to have

the HI cline center: 28 were shifted significantly west and 15 were shifted significantly east (Appendix 2.1). Similarly, 57% (58/102) could not be constrained to have the hybrid index cline width: 42 were significantly wider and 16 were significantly narrower (Appendix 2.1).

Dispersal and selection in the hybrid zone

The mark-recapture method yielded a direct estimate for RMS dispersal distance of 0.75 km. Using this estimate of dispersal combined with a time since secondary contact of 21 kya yielded a neutral cline width of 272.8 km. Both hybrid index clines and 95/102 SNP clines had widths with CIs that encompassed this value. However, seven SNP clines were significantly narrower than the cline predicted by neutral diffusion. Effective selection on the hybrid index cline was very low: 0.007 (0.002-0.03)%. Across all SNPs there was substantial variation in effective selection pressure (mean = 0.08 ± SD 0.35%, range = $4x10^{-5}$-3%).

Comparing genetic and plumage clines

For plumage hue, the best-fitting model did not estimate mean or variance at the cline ends and modeled both sets of tail parameters separately (Table 2.2). This cline had a center of 2,110.5 (2,095.5-2,129.1) km, which was 357.3 km east of the HI center, 388.8 km east of the average SNP cline center, and located 4 km west of sampling location 11 (Figures. 2.3, 2.4). The width of the plumage cline was 376.2 (286.6-511.2) km. The CI for the plumage cline center did not overlap with CIs for the HI, HI_{diag}, or average SNP cline centers. Comparing CIs between the center of the plumage cline and individual SNP loci clines revealed that the plumage cline was shifted significantly east relative to 88% (90/102) of the SNP clines. Seven SNP clines had centers coincident with the plumage cline, and five had centers shifted significantly east of the plumage cline. No SNP cline had the same overall shape as the plumage cline, as the seven SNP clines with centers coincident with the plumage cline were much wider than the plumage cline (Figure 2.3). Constraining the center of the plumage cline to have the either the HI or HI_{diag} cline center produced a significantly worse fit (ΔAICc = 56.5 and 71.3 respectively, Table 2.3).

Discussion

Characteristics of the hybrid zone

The hybrid zone as determined by genetic markers is centered at the Carpentarian Barrier, and is likely a result of secondary contact following allopatric genetic and morphological divergence, as suggested by Lee and Edwards (2008). Overall, 58% and 43% of the SNPs had the same cline center and width, respectively, as the hybrid index cline, indicating that there is considerable genome-wide coincidence and concordance at the Carpentarian Barrier. Both hybrid indices and 61% of the SNP clines best fit a symmetrical model with no tail parameters, indicating negligible introgression outside of the center. These patterns are consistent with a tension zone where multiple unlinked loci form steep coincident clines at the hybrid zone center (Barton 1983; Barton and Hewitt 1985; Barton and Gale 1993). However, the fact that many of the individual SNP clines could not be constrained to have the same center or width suggests that the extent of introgression across the hybrid zone varies by locus. Of the SNP clines that did not have the same center as the hybrid index cline, most were small shifts of less than one cline width to either side. These staggered clines may be due to selection against certain deleterious allele combinations, but especially in cases where the cline center is displaced but there is a long tail of introgression, drift or variance due to missing data are more likely explanations (Barton 1983; 1993; Polechova and Barton 2011). This genomic dataset contains SNPs that appear to exhibit both of these patterns, as cline shape and thus the extent of introgression outside of the zone center, was highly variable among loci. According to the tension zone model, selection against hybrids likely explains the pattern seen in the 16 SNPs that had significantly narrower cline widths than the hybrid index. These loci may be located in genomic regions that contribute to reproductive isolation or local adaptation (Harrison 1990; Wu 2001). In contrast, recombination among alleles of the 58 SNPs with significantly wider cline widths appears to be relatively unrestricted, likely due to a low level of linkage with loci under selection, resulting in bi-directional introgression across the hybrid zone.

Hybrid zone maintenance

Selection against hybrids appears to be very weak in this system, as the hybrid index had a width statistically similar to the cline predicted by neutral diffusion. In fact, 93% of the SNP clines were wider than the neutral cline, indicating that overall introgression is relatively unrestricted. It is important to note that these inferences depend heavily on an accurate measure of dispersal distance. My direct measure of 0.75 km is likely an underestimate due to the limited size of the study area and the inability to detect long distance migrants or account for historic colonization events that would result in higher effective dispersal (Barton and Gale 1993). Nonetheless, even with this conservative estimate of dispersal, seven SNP clines were significantly narrower than the neutral cline, suggesting that selection in certain parts of the genome is strong enough to restrict introgression.

Analysis of effective selection in the hybrid zone also suggests that overall selection is low but variable depending on the locus. My estimate of average effective selection on a locus of 0.08% (range = $4x10^{-5}$-3%) is lower than most published studies of other hybrid zones (e.g., *Heliconius* butterflies = 20-30%, Mallet et al. 1990; *Bombina* toads = 17-22%, Szymura and Barton 1991; *Carlia* skinks = 22-70%, Phillips et al. 2004; *Mus* house mice = 6-9%, Macholán et al. 2007; *Vandiemenella* grasshoppers = 6-41%, Kawakami et al. 2009). While it may be true that average selection in the hybrid zone between subspecies of the red-backed fairy-wren is lower than between these more highly diverged taxa, I do not place too great an emphasis on the quantitative values because they are so heavily dependent on an accurate measure of dispersal. Because my estimate of dispersal is a conservative one, the values for effective selection derived from it should be considered a lower limit, and the actual strength of selection is likely higher. Furthermore, the total fitness reduction of hybrids is likely to be many times higher than the effective selection pressure on a particular locus.

In addition to the results from the geographic cline analysis of genetic markers presented here, there are also several other lines of evidence supporting the tension zone model. Playback

experiments have shown that territorial males are significantly more likely to respond to their own subspecies' song, and that variation in song is strongly bimodal and coincident with the genetic hybrid zone (Greig and Webster 2013), suggesting it is a social signal functioning as a speciation phenotype (*sensu* Shaw and Mullen 2011). Additionally, although the Carpentarian Barrier was most prominent as a barrier to dispersal during the Pleistocene, the area is currently sparsely vegetated, frequently flooded, and supports a relatively low density of red-backed fairy-wrens (Chivas et al. 2001; D. Baldassarre, pers. obs.), and tension zones often stabilize in areas of low population density (Barton 1979a; Barton and Hewitt 1985; 1989). Together these observations suggest that the hybrid zone may be located in an area where extrinsic social and ecological selection results in a hybrid disadvantage.

Asymmetrical introgression of plumage color

The geographic cline analyses support the hypothesis that alleles for red plumage color have introgressed asymmetrically across the hybrid zone following secondary contact, as the plumage cline was displaced significantly east of the hybrid index clines and the vast majority of the individual SNP clines (Figures. 2.3, 2.4). Five SNP clines had centers located east of the plumage cline, supporting the idea that eastward genetic introgression in some parts of the genome could have been substantial enough to recombine "red alleles" from the *cruentatus* subspecies into the genomic background of the orange *melanocephalus* subspecies. Chapter 3 details experiments showing that experimentally reddened males in an allopatric population of the *melanocephalus* subspecies sired significantly more extra-pair offspring than orange males (Baldassarre and Webster 2013). The greater reproductive success of red males likely provides the selective force driving the observed introgression, eroding one potential barrier between the subspecies. This hybrid zone appears to be a rare example of the potential for hybridization to introduce adaptive traits into an alternate environment and genomic background due to a sexually selected advantage (but see Stein and Uy 2006). Introgression of a sexual trait from one parental taxon into the other across a hybrid zone can thus eliminate one potential speciation

phenotype (*sensu* Shaw and Mullen 2011), and restrict, rather than promote, the speciation process (Servedio et al. 2013).

There are several alternative explanations for the lack of coincidence between the genetic and plumage clines. First, variation in plumage color may not have a strong genetic basis, but rather be determined predominately by environmental variation due to, for example, dietary intake of carotenoids (Hill 1992). Under this scenario, the plumage contact zone may lie on an ecotone that happens to occur in an area other than the location of secondary contact. I have argued elsewhere that this is unlikely due to the fact that variation in plumage color is not well explained by environmental variation, but rather follows a clear pattern of isolation by distance, consistent with strong genetic underpinnings (Chapter 1; Baldassarre et al. 2013). Second, the plumage contact zone may represent the true location of secondary contact, and alleles at the SNP loci analyzed here may have subsequently introgressed westward. Under this scenario, we would expect to find many SNP clines that share the same center as the plumage cline, but my geographic cline and STRUCTURE results refute this idea, corroborating the results from Lee and Edwards (2008) that there is no significant genetic structure across the plumage contact zone. Additionally, the Carpentarian Barrier is older than any biogeographic barrier along the east coast (Cracraft 1986). Coupled with the age of the divergence between subspecies (Lee and Edwards 2008), secondary contact at the Carpentarian Barrier followed by asymmetrical introgression of red plumage color is a more likely explanation.

Finally, plumage color may be selectively neutral, and the displaced cline may be due to genetic dominance of red over orange alleles. This alternative is more difficult to eliminate due to ignorance of the genetic architecture of plumage color in this species, but has been invoked to explain displaced plumage clines in hybrid zones between *Gymnorhina* magpie (Hughes 1982) and *Setophaga* warbler (Rohwer and Wood 1998) species pairs. In the red-backed fairy-wren, there is experimental evidence indicating that red plumage color is not neutral and is in fact under strong sexual selection on the eastern side of the hybrid zone (Chapter 3; Baldassarre and Webster 2013). However, genetic dominance may still contribute to the displaced cline

depending on how many loci are involved. If red plumage were dominant over orange plumage, orange males would be most likely to occur just west the plumage cline center, near the source of *melanocephalus* alleles. Because plumage hue was measured on a continuous scale, it is difficult to determine the true frequency of "pure orange" birds at this location, but if I consider a bird with a plumage hue value that overlaps with the distribution of hues in the farthest allopatric *melanocephalus* population (sampling location 16), there are no orange birds west of the plumage cline center. However, at sampling location 10, located at the plumage cline center, the frequency of orange birds is 1/11 = 0.09 (Figure 2.5). If I further assume that the frequency of orange alleles in this population is equal to the average *melanocephalus* SNP allele frequency of 0.86, then eight unlinked loci would be required to produce the observed phenotypic frequency ($[(0.86)^2]^8 = 0.09$). Although this inference is based on a relatively small sample size, it is substantially higher than the number of genes coding for plumage color in other species (Johnson and Brush 1972; Rohwer and Wood 1998; Domyan et al. 2014). There may also be epigenetic effects that affect plumage color that would not be explained by simple underlying allele frequencies. However, without more detailed study of the genetics of plumage color or crossing experiments, the likelihood of these alternatives is difficult to evaluate. Thus, the most likely explanation is that the displaced plumage cline is the result of asymmetrical introgression of alleles for red plumage driven by sexual selection, with possible contributions from genetic dominance and epigenetic effects.

Traits subject to positive selection on one side of a hybrid zone are expected to quickly sweep to fixation (Barton 1979b; Hewitt 1988), but red plumage color has not yet done so. The red-backed fairy-wren is sedentary and exhibits limited dispersal (Varian-Ramos and Webster 2012), particularly relative to the spatial scale of the transect analyzed here, likely limiting the speed of introgression. The spread of these alleles may be further slowed if they are sex-limited to males, as dispersal is female-biased in this species (Varian-Ramos and Webster 2012). It is also possible that delayed plumage maturation – where male nuptial plumage is often not expressed until at least the second breeding year (Karubian et al. 2008) – may slow the spread of

the signal. Additionally, farther eastward introgression may be limited by another zoogeographic barrier, the Burdekin Gap (Galbraith 1969; Cracraft 1986), which is a large area of arid habitat located just south of sampling location 13 (Figure 2.1) that separates the northern and central Queensland forest biomes, and is known to be a dispersal barrier for several taxa (Chapple et al. 2011). The leading edge of the plumage cline is also located at this area, 2,317 km along the transect (Figures 2.3-2.5). Finally, although field experiments have demonstrated a strong mating advantage for red males in an allopatric orange population (Chapter 3; Baldassarre and Webster 2013), the effect of plumage color on fitness in the center of the plumage cline is unknown. If there is relaxed selection on plumage color in the contact zone, this could slow introgression and may also explain the relatively large estimated width of the plumage cline.

Conclusions

In summary, I used a large multi-locus SNP dataset combined with reflectance spectrophotometry to analyze genetic and plumage color variation across a hybrid zone between two subspecies of the red-backed fairy-wren. I found coincident and concordant clines for many putatively unlinked SNPs centered at the Carpentarian Barrier, a region previously hypothesized to be the location of secondary contact (Lee and Edwards 2008). Although some SNP clines varied greatly in both center and width, several clines were narrower than would be predicted by neutral diffusion, suggesting the hybrid zone is a tension zone maintained by a balance of dispersal and weak selection against hybrids. Analysis of the cline for plumage color suggested asymmetrical introgression of red plumage into the genetic background of the orange subspecies, consistent with strong sexual selection on red males east of the hybrid zone (Chapter 3; Baldassarre and Webster 2013). The displaced plumage cline may also be affected by genetic dominance of red alleles or epigenetic effects. The red-backed fairy-wren hybrid zone thus exemplifies the potential for sexual selection to erode rather than create a potential reproductive barrier between taxa (Servedio et al. 2013). This study highlights the importance of analyzing hybrid zones between taxa subject to strong sexual selection at an intermediate stage of

divergence, as these systems provide unique opportunities to examine the possibility that sexual selection can promote adaptive introgression. Furthermore, incorporating genomic and morphological data into analyses will increase our understanding of differential introgression across the genome.

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Table 2.1 The sampling transect across the red-backed fairy-wren species range. Variation in hybrid index and plumage hue scores is presented as standard deviations. Certain sampling locations were omitted from one of the datasets if no nuptially-plumaged males were captured (location 5), they were sampled by a collaborator after genomic analyses began (location 2), or they were located particularly close to another location included in the dataset (locations 9 and 10).

No.	Name	Dist. (km)	Ancestry	HI	HI_{diag}	Plumage hue	Gen. *N*	Plum. *N*
1	Mornington	0	*cruent*	0.999 ± 0.001	0.999 ± 0.001	0.926 ± 0.054	15	10
2	Darwin	720	*cruent*	N/A	N/A	0.906 ± 0.069	0	15
3	Camooweal	1,293	*cruent*	0.999 ± 0.004	0.997 ± 0.005	0.798 ± 0.121	15	8
4	Mt. Isa	1,450	*cruent*	0.991 ± 0.024	0.992 ± 0.027	0.774 ± 0.089	15	7
5	Croydon	1,710	admixed	0.635 ± 0.037	0.571 ± 0.092	N/A	9	0
6	Georgetown	1,810	admixed	0.325 ± 0.082	0.224 ± 0.107	0.735 ± 0.078	15	6
7	Mt. Surprise	1,930	*melano*	0.043 ± 0.064	0.028 ± 0.065	0.725 ± 0.067	15	6
8	Donkey Farm	2,045	*melano*	0.016 ± 0.018	0.011 ± 0.013	0.655 ± 0.091	10	27
9	Moomin	2,051	*melano*	N/A	N/A	0.649 ± 0.124	0	25
10	Ravenshoe	2,053	*melano*	N/A	N/A	0.598 ± 0.123	0	10
11	Cardwell	2,114	*melano*	0	0.001 ± 0.001	0.505 ± 0.148	14	11
12	Paluma	2,139	*melano*	0	0.001 ± 0.001	0.424 ± 0.101	15	8
13	Cromarty	2,220	*melano*	0.001 ± 0.001	0.002 ± 0.002	0.307 ± 0.11	11	8
14	Eungella	2,386	*melano*	0.001 ± 0.001	0.001 ± 0.001	0.236 ± 0.142	13	7
15	Taunton	2,527	*melano*	0.001 ± 0.002	0.002 ± 0.002	0.146 ± 0.095	10	7
16	Brisbane	3,052	*melano*	0.001 ± 0.001	0.001 ± 0.001	0.087 ± 0.058	12	23
						Total	169	178

Table 2.2 Parameter estimates for the best-fitting hybrid index and plumage hue clines. Two log-likelihood unit support limits are presented in parentheses. Cline width (w) is 1/maximum slope, cline center (c) is measured in distance (km) from sampling location 1, p_{min} and p_{max} are the allele frequencies at the ends of the cline, and δ and τ are the shape parameters for the left and right tails.

Trait	c	w	P_{min}	P_{max}	δ_L	τ_L	δ_R	τ_R
HI	1,753.2 (1,673.9-1,813.2)	251.3 (131.7-451.6)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
Hi_{diag}	1,730.4 (1,648.5-1,790.7)	238.5 (114.4-438.8)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
Plumage hue	2,110.5 (2,095.5-2,129.1)	376.2 (286.6-511.2)	N/A	N/A	64.4 (39.9-124.8)	0.1 (0.0-0.2)	69.1 (3.7-171.4)	0.4 (0.3-0.6)

Table 2.3 AICc scores for the free or constrained plumage hue clines. The plumage hue cline was constrained to have either the HI or HI_{diag} cline center.

Model	*c*	AICc
Unconstrained	2,110.5	-309.1
HI-constrained	1,753.2	-252.6
Hi_{diag}-constrained	1,730.4	-237.8

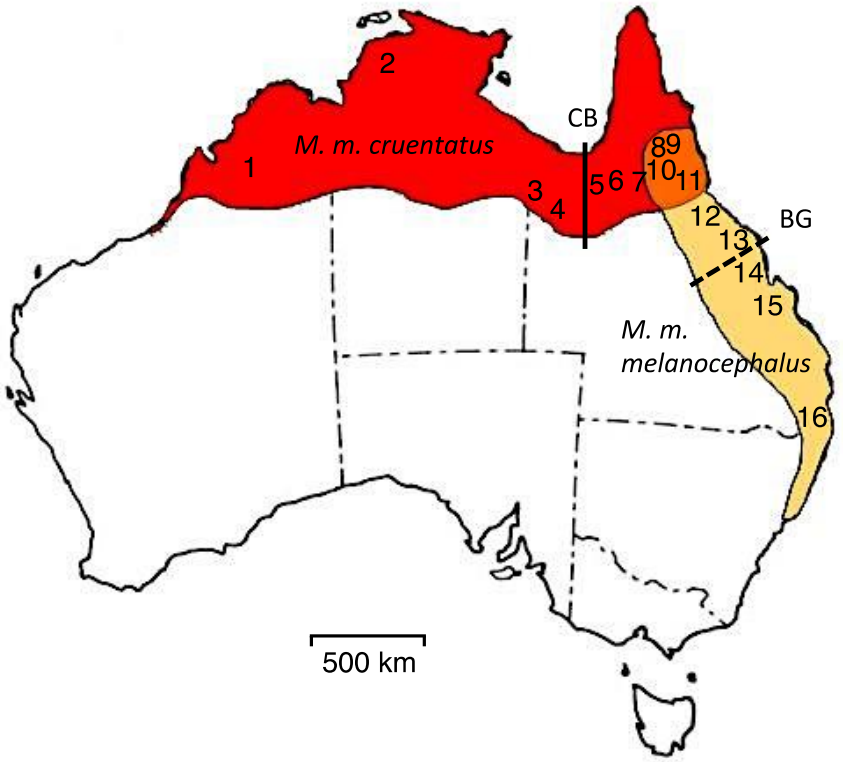

Figure 2.1 The species range of the red-backed fairy-wren showing the currently recognized distribution of the red *cruentatus* subspecies in the west and the orange *melanocephalus* subspecies in the east. The intermediately colored area in the northeast represents the hypothesized region of plumage overlap based on subjective evaluation of plumage color in the field. The solid line represents the Carpentarian Barrier (CB) and the dashed line represents the Burdekin Gap (BG). Numbers refer to sampling locations along the transect.

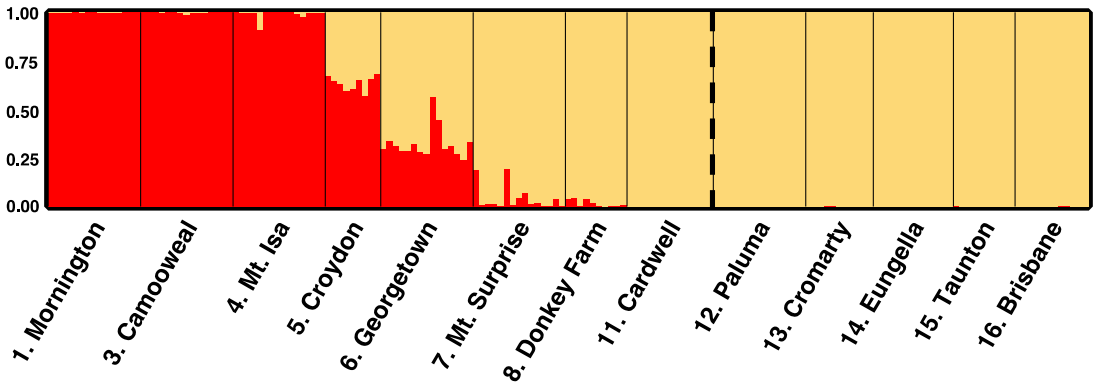

Figure 2.2 Results of a Bayesian analysis of assignment probability (on the y-axis) to either the western *cruentatus* genetic cluster (red) or the eastern *melanocephalus* genetic cluster (orange) using the program STRUCTURE with the 2,702 loci dataset. Results are not shown for the 102 diagnostic loci dataset because they were qualitatively the same. Each colored vertical bar represents an individual, with black bars separating sampling locations. The dashed vertical line represents the approximate location of the plumage contact zone.

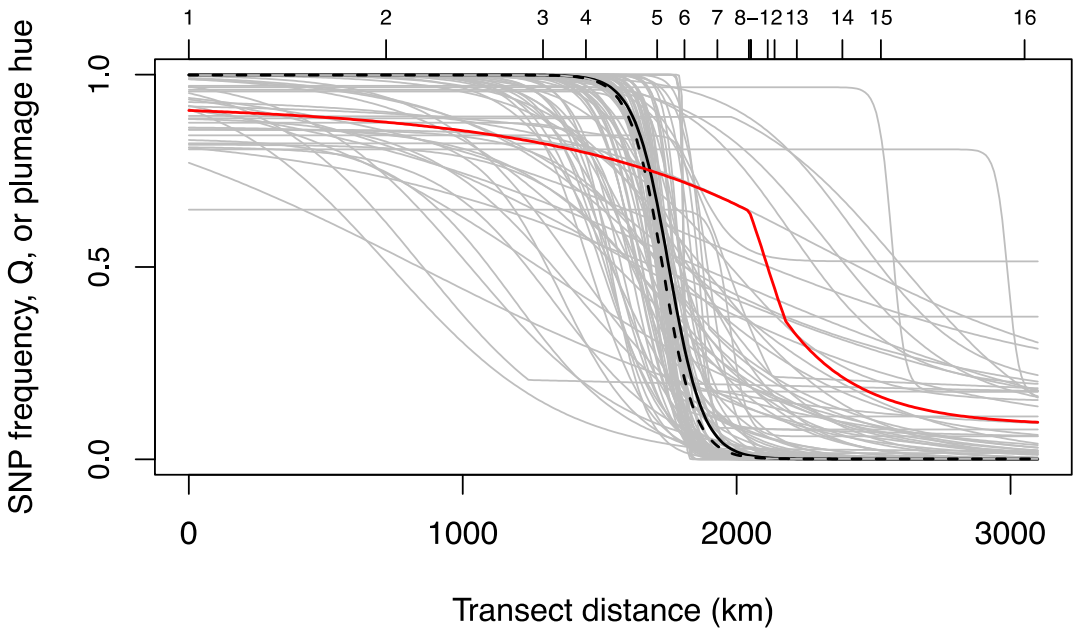

Figure 2.3 Combined maximum likelihood geographic clines for the 2,702 loci hybrid index (HI, black), the 102 diagnostic loci hybrid index (HI_{diag}, black dashed), each of the 102 diagnostic SNP loci (grey), and plumage hue (red). Higher values for hue correspond to redder plumage color. All SNP loci have one allele with frequency > 0.8 on one end of the cline and < 0.2 on the other end, even those with very wide fitted clines that appear to deviate from this criterion. Numbers along the top refer to sampling locations.

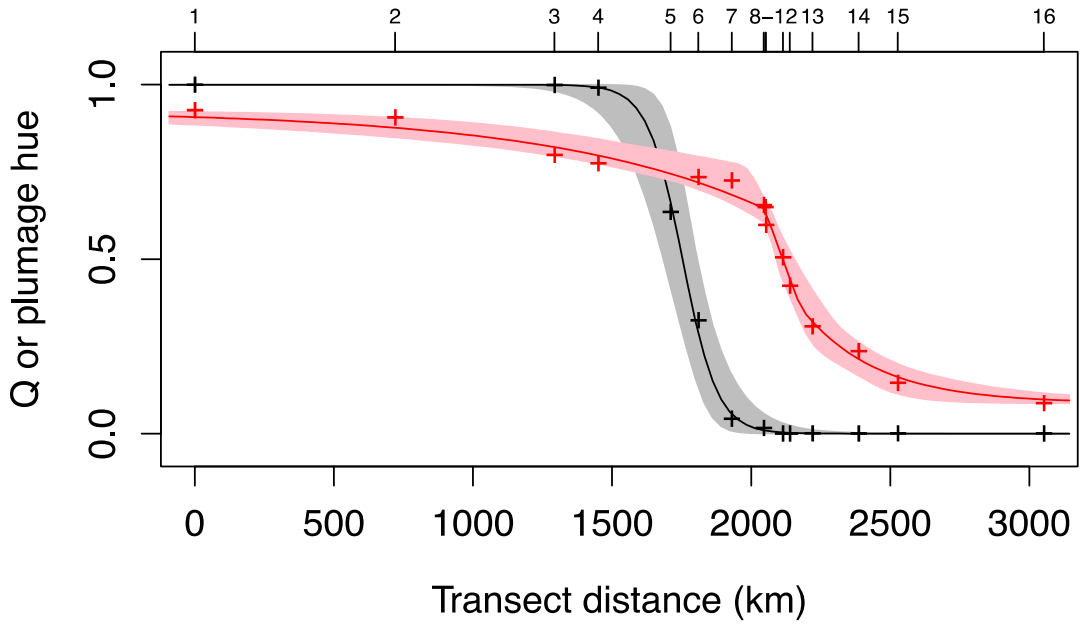

Figure 2.4 The maximum likelihood cline and the 95% credible cline region for the 2,702 loci hybrid index (HI, black) and plumage hue (red). Higher values for hue correspond to redder plumage color. Numbers along the top refer to sampling locations.

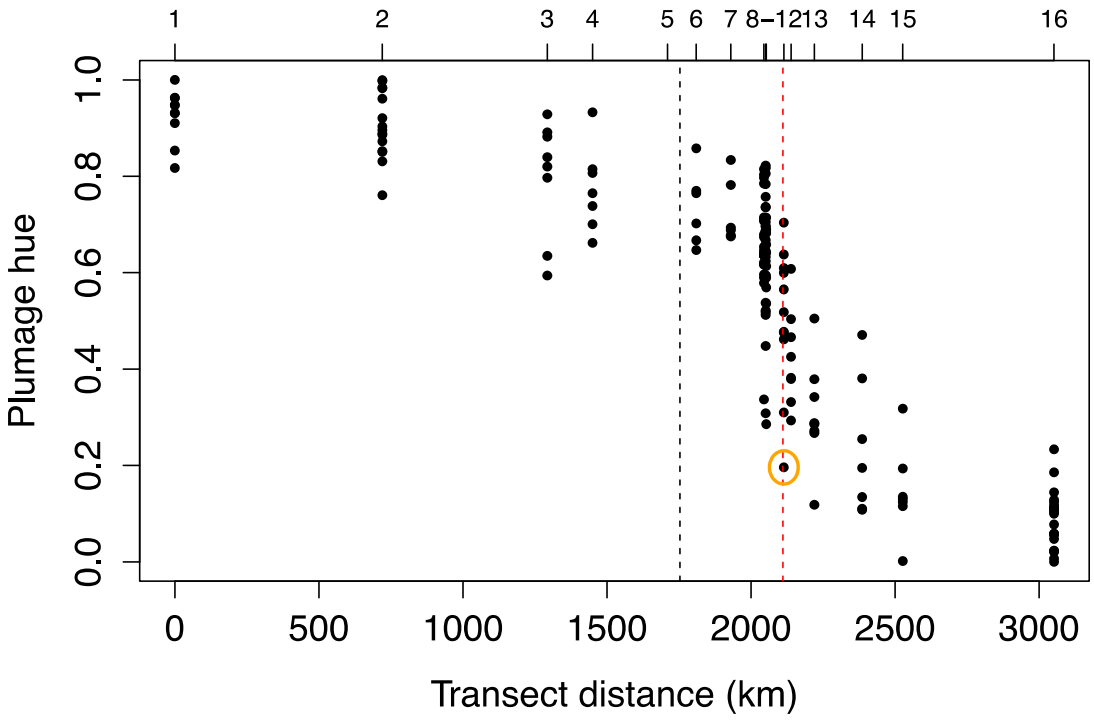

Figure 2.5 Variation in plumage hue across the transect, with the hybrid index (black) and plumage cline (red) centers marked with dashed lines. Higher values for hue correspond to redder plumage color. At sampling location 11, at the center of the plumage cline, the frequency of orange birds is 1/11 = 0.09 (represented by the circled point). Eight unlinked loci would be required for genetic dominance of red plumage alleles to explain this phenotypic frequency (see text for details). Numbers along the top refer to sampling locations.

Appendix 2.1 Best-fitting maximum likelihood cline model parameters for all individual SNP loci, as well as results of analyses of concordance with the genomic hybrid index cline. Values in parentheses are two log-likelihood unit support limits for each parameter estimate. If a given SNP cline center or width deviated significantly from the hybrid index cline, it is indicated in bold.

Locus	Best-fitting model	*c*	*w*	*Pmin*	*Pmax*	*δL*	*τL*	*δR*	*τR*
1	*Pmin*/*Pmax* fixed, no tails	1774.7 (1693-1839.5)	309.3 (190.1-507.2)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
2	*Pmin*/*Pmax* fixed, no tails	1730.8 (1645.4-1789.9)	206 (101.3-400.1)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
3	*Pmin*/*Pmax* fixed, no tails	1705.1 (1618.4-1757.7)	165.3 (68.9-354.3)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
4	*Pmin*/*Pmax* fixed, no tails	**1839.4 (1789.2-1886.4)**	154.3 (82.5-295.9)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
5	*Pmin*/*Pmax* obs., mirror tails	1806 (1690.3-1899.3)	**255.1 (113.4-917.8)**	0.1 (fixed)	0.9 (fixed)	124.2 (1.3-827.6)	0.1 (0.0-1.0)	124.2 (1.3-827.6)	0.1 (0.0-1.0)
6	*Pmin*/*Pmax* observed, no tails	**2428.7 (2256.8-2746.2)**	**835.1 (469.1-1659.1)**	0.2 (fixed)	1.0 (fixed)	none	none	none	none
7	*Pmin*/*Pmax* fixed, no tails	1866.1 (1528.4-2207.4)	**3249.9 (2144.6-3251.9)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
8	*Pmin*/*Pmax* fixed, no tails	**1530.8 (1444-1620.1)**	345.3 (204.2-578.5)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
9	*Pmin*/*Pmax* observed, no tails	1807 (1426.4-2133.9)	**2645.8 (1428.2-3251.7)**	0.1 (fixed)	0.9 (fixed)	none	none	none	none
10	*Pmin*/*Pmax* fixed, no tails	**1557 (1468.7-1643.9)**	376 (242-596.1)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
11	*Pmin*/*Pmax* fixed, no tails	**736.3 (-99.7-1200.3)**	**1272.3 (598.7-2955)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
12	*Pmin*/*Pmax* estimated, no tails	1711.4 (1456.4-2010.8)	**21 (0.4-1732.6)**	0.4 (0.1-0.5)	0.9 (0.8-1.0)	none	none	none	none
13	*Pmin*/*Pmax* observed, no tails	**1185.7 (550.2-1404.2)**	**867.5 (446.2-2075.7)**	0.0 (fixed)	0.8 (fixed)	none	none	none	none
14	*Pmin*/*Pmax* fixed, no tails	1717.4 (1633.7-1772.4)	187.8 (89.9-370.3)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
15	*Pmin*/*Pmax* fixed, no tails	1740.8 (1662.2-1801.8)	277.1 (159.8-466.6)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
16	*Pmin*/*Pmax* fixed, no tails	1727.5 (1656.7-1771.8)	143.4 (67.4-319.5)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
17	*Pmin*/*Pmax* fixed, no tails	**1627.9 (1523.1-1719.1)**	**474.2 (320.8-724.1)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none

18	*Pmin*/*Pmax* fixed, no tails	1726.8 (1642.1-1787.9)	238.4 (125.4-431.3)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
19	*Pmin*/*Pmax* estimated, no tails	1869 (1810.6-1928.4)	**100.2 (0-250.1)**	0.0 (0.0-0.1)	0.8 (0.7-0.9)	none	none	none	none
20	*Pmin*/*Pmax* fixed, no tails	**1641.5 (1553.9-1719.4)**	313.9 (194.3-505.1)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
21	*Pmin*/*Pmax* estimated, no tails	**2989.8 (2468.7-3151.6)**	**85.8 (0.1-1944.5)**	0.2 (0.0-0.7)	0.8 (0.7-0.9)	none	none	none	none
22	*Pmin*/*Pmax* fixed, no tails	1762.3 (1670.1-1839.4)	390.1 (244.7-616.5)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
23	*Pmin*/*Pmax* fixed, no tails	**1580.1 (1495.7-1663.4)**	327.7 (209.2-524.4)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
24	*Pmin*/*Pmax* observed, no tails	**1358.7 (1108-1496.7)**	596.2 (325.1-1203.7)	0.0 (fixed)	0.9 (fixed)	none	none	none	none
25	*Pmin*/*Pmax* fixed, no tails	1716.8 (1635.3-1770.9)	193.6 (94.3-378.2)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
26	*Pmin*/*Pmax* fixed, no tails	1843.8 (1731.2-1941.8)	**721.3 (500.1-1093.7)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
27	*Pmin*/*Pmax* fixed, no tails	**840.5 (248-1317.8)**	**2774.1 (1611-3251.7)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
28	*Pmin*/*Pmax* observed, no tails	**1396.7 (1003.2-1588.4)**	**1053 (645.6-2056.2)**	0.0 (fixed)	0.9 (fixed)	none	none	none	none
29	*Pmin*/*Pmax* observed, no tails	1713.8 (1476.7-1887.9)	**1475 (946.3-2587.1)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
30	*Pmin*/*Pmax* observed, no tails	1826.5 (1403.8-2228.4)	**2869.9 (1489.3-3252)**	0.2 (fixed)	1.0 (fixed)	none	none	none	none
31	*Pmin*/*Pmax* observed, no tails	**2193.8 (2057.9-2391.1)**	**797.9 (426.8-1506.2)**	0.2 (fixed)	1.0 (fixed)	none	none	none	none
32	*Pmin*/*Pmax* fixed, no tails	1738 (1660.8-1784.5)	146.2 (70.4-343.6)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
33	*Pmin*/*Pmax* fixed, no tails	1728.1 (1642.4-1780.4)	160.8 (75.4-353.8)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
34	*Pmin*/*Pmax* fixed, no tails	**1548.2 (1445.2-1641.1)**	**488.7 (328.9-755.2)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
35	*Pmin*/*Pmax* fixed, no tails	**1653.7 (1569.8-1727.6)**	284.1 (169.5-459.1)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
36	*Pmin*/*Pmax* observed, no tails	1974.6 (1786.9-2155.2)	**864.7 (381.8-1964.6)**	0.2 (fixed)	0.9 (fixed)	none	none	none	none
37	*Pmin*/*Pmax* fixed, no tails	1766.7 (1693.3-1825.3)	266.8 (153.7-456.6)	0.0 (fixed)	1.0 (fixed)	none	none	none	none

38	*Pmin*/*Pmax* fixed, no tails	1712.6 (1631.6-1761.8)	156.2 (67.1-344.1)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
39	*Pmin*/*Pmax* fixed, right tail	**854.4 (158.6-1184.1)**	**1145.6 (207.5-2831.1)**	0.0 (fixed)	1.0 (fixed)	none	none	386.2 (0.2-929.7)	0.0 (0.0-0.8)
40	*Pmin*/*Pmax* fixed, right tail	1734.8 (1588.9-1831)	**147.3 (35.1-549.5)**	0.0 (fixed)	1.0 (fixed)	none	none	24.9 (0-296.2)	0.1 (0.0-0.8)
41	*Pmin*/*Pmax* fixed, no tails	1808.6 (1760.5-1834.2)	12.3 (1-213.6)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
42	*Pmin*/*Pmax* fixed, right tail	1747.1 (1534.1-1809.2)	**300 (42.5-542.3)**	0.0 (fixed)	1.0 (fixed)	none	none	181 (0-349.5)	0.0 (0.0-0.5)
43	*Pmin*/*Pmax* obs., right tail	**1769.2 (1755.9-1899.9)**	**111.1 (111.1-616.5)**	0.1 (fixed)	1.0 (fixed)	none	none	4.2 (1.0-106.0)	0.1 (0.0-0.6)
44	*Pmin*/*Pmax* fixed, no tails	1740.3 (1685.2-1780)	123.7 (62.4-282.7)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
45	*Pmin*/*Pmax* fixed, no tails	**1633.9 (1547.4-1711.8)**	277.9 (164.9-459.9)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
46	*Pmin*/*Pmax* fixed, no tails	**1540.1 (1464.8-1625.4)**	271 (151.7-464.6)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
47	*Pmin*/*Pmax* fixed, no tails	1794.1 (1744.4-1836.6)	141.7 (67-290.8)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
48	*Pmin*/*Pmax* fixed, no tails	**1808.9 (1773.2-1838.9)**	**25.1 (1-204.2)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
49	*Pmin*/*Pmax* fixed, no tails	1794.1 (1751.4-1831.5)	109.8 (43.7-252.4)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
50	*Pmin*/*Pmax* fixed, no tails	1739.7 (1652.5-1799.8)	220.9 (111.6-420.9)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
51	*Pmin*/*Pmax* fixed, no tails	**1027.2 (405.7-1310.5)**	**1194.7 (666-2359)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
52	*Pmin*/*Pmax* fixed, right tail	1678.2 (1483.7-1806.1)	**304.8 (99.3-739.9)**	0.0 (fixed)	1.0 (fixed)	none	none	79.4 (0.1-572.6)	0.2 (0.0-1.0)
53	*Pmin*/*Pmax* fixed, mirror tails	**2605.7 (2412.7-3151.5)**	**1192.6 (168.2-2992.9)**	0.0 (fixed)	1.0 (fixed)	622.8 (51.1-1456.0)	0.0 (0.0-0.5)	622.8 (51.1-1456)	0.0 (0.0-0.5)
54	*Pmin*/*Pmax* observed, no tails	**1336.2 (853.1-1536.2)**	**859.8 (481.1-1944.8)**	0.0 (fixed)	0.8 (fixed)	none	none	none	none
55	*Pmin*/*Pmax* fixed, no tails	**1543.4 (1362-1663.9)**	**653.6 (425-1111.8)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
56	*Pmin*/*Pmax* fixed, right tail	**1876.8 (1821.6-2005.1)**	**137.8 (80.4-540.8)**	0.0 (fixed)	1.0 (fixed)	none	none	22.9 (0.7-296.3)	0.1 (0.0-0.9)
57	*Pmin*/*Pmax* fixed, no tails	**1807 (1764.5-1827.5)**	**22 (1.2-197.2)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none

58	*Pmin*/*Pmax* fixed, no tails	**1413.9 (1101.4-1582.1)**	**1001.3 (639.1-1796.8)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
59	*Pmin*/*Pmax* fixed, no tails	1792.7 (1699.6-1874.7)	**507.4 (344.4-764.2)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
60	*Pmin*/*Pmax* fixed, no tails	**1686.1 (1601-1737.9)**	165.6 (65.1-342.4)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
61	*Pmin*/*Pmax* estimated, no tails	**1841.1 (1678.3-2162.2)**	**310.2 (3.1-1176.9)**	0.5 (0.2-0.7)	1.0 (0.9-1.0)	none	none	none	none
62	*Pmin*/*Pmax* fixed, no tails	1813 (1576.1-2004.6)	**1630.1 (1057-2983.3)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
63	*Pmin*/*Pmax* fixed, no tails	1875.9 (1723.4-2014)	**1144.9 (783.4-1865.9)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
64	*Pmin*/*Pmax* fixed, no tails	**1894.8 (1838.9-1947.5)**	204.2 (118.5-356.5)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
65	*Pmin*/*Pmax* fixed, no tails	**1656 (1567.9-1730)**	276.3 (156.2-466.6)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
66	*Pmin*/*Pmax* estimated, no tails	1732.7 (1485.9-1822.9)	**249.9 (10.6-885.7)**	0.1 (0.0-0.2)	0.8 (0.7-1.0)	none	none	none	none
67	*Pmin*/*Pmax* estimated, no tails	**2566.8 (2498.6-3151.4)**	**95.4 (0.1-1068)**	0.2 (0.0-0.7)	1.0 (0.9-1.0)	none	none	none	none
68	*Pmin*/*Pmax* fixed, no tails	**1520.8 (1425.2-1611.4)**	420.3 (269.3-672.7)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
69	*Pmin*/*Pmax* fixed, no tails	1770.8 (1722.3-1809.8)	119.6 (58.8-269.3)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
70	*Pmin*/*Pmax* fixed, right tail	1733.9 (1603.9-1873.7)	**241.6 (123.9-731.2)**	0.0 (fixed)	1.0 (fixed)	none	none	8.5 (0.1-292.7)	0.3 (0.0-1.0)
71	*Pmin*/*Pmax* fixed, no tails	1721.8 (1649.6-1768)	159.3 (75.2-337.8)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
72	*Pmin*/*Pmax* fixed, no tails	**1464.4 (1355.9-1557.1)**	445.9 (273.6-753.6)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
73	*Pmin*/*Pmax* fixed, no tails	1676.1 (1541.3-1785.1)	**630.3 (419.5-1005.6)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
74	*Pmin*/*Pmax* fixed, no tails	**1278.7 (727.5-1569.7)**	**2105.9 (1292.8-3251.9)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
75	*Pmin*/*Pmax* observed, no tails	1790.7 (1629.3-1921.1)	**824.5 (516-1416.3)**	0.0 (fixed)	0.9 (fixed)	none	none	none	none
76	*Pmin*/*Pmax* fixed, no tails	1685.3 (1599.1-1753.6)	266.3 (147-452.5)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
77	*Pmin*/*Pmax* observed, no tails	**2305.3 (2013.6-2819.8)**	**2074.1 (1100.2-3251.8)**	0.2 (fixed)	0.9 (fixed)	none	none	none	none

78	*Pmin*/*Pmax* fixed, right tail	**1964.6 (1832.7-2049.8)**	**516 (149.3-847.9)**	0.0 (fixed)	1.0 (fixed)	none	none	167.4 (0.1-362.2)	0.0 (0.0-0.6)
79	*Pmin*/*Pmax* observed, no tails	1709.6 (1488.2-1866.8)	**1064.8 (672.6-1884.2)**	0.0 (fixed)	0.9 (fixed)	none	none	none	none
80	*Pmin*/*Pmax* fixed, right tail	1757.8 (1695.1-1803.1)	144.3 (23.9-334.8)	0.0 (fixed)	1.0 (fixed)	none	none	127.7 (8.3-353.6)	0.0 (0.0-0.3)
81	*Pmin*/*Pmax* fixed, no tails	1807.1 (1763.2-1829.6)	**20.3 (0.4-201.7)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
82	*Pmin*/*Pmax* fixed, no tails	1718.1 (1627-1792)	333.9 (200.2-543.1)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
83	*Pmin*/*Pmax* fixed, no tails	1734 (1672.3-1775.3)	120.4 (55.7-293.7)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
84	*Pmin*/*Pmax* fixed, no tails	**1507 (1389.3-1604.8)**	476.8 (305.3-784.8)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
85	*Pmin*/*Pmax* fixed, no tails	1825.7 (1679.7-1951.8)	**947.2 (642.5-1527)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
86	*Pmin*/*Pmax* fixed, no tails	1641.5 (1512.6-1749.6)	**578.1 (386.2-912.6)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
87	*Pmin*/*Pmax* fixed, right tail	1805.4 (1715.9-1882.2)	**235.5 (83.1-586.9)**	0.0 (fixed)	1.0 (fixed)	none	none	92.4 (0.4-458.3)	0.1 (0.0-1.0)
88	*Pmin*/*Pmax* estimated no tails	1737.9 (1653.9-1805.9)	**96.4 (0.1-459)**	0.2 (0.1-0.3)	1.0 (0.8-1.0)	none	none	none	none
89	*Pmin*/*Pmax* fixed, no tails	1707.6 (1623.8-1768.9)	226.2 (114.8-411.8)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
90	*Pmin*/*Pmax* observed, no tails	1727.3 (1539.8-1868.6)	**906.7 (581.4-1545.9)**	0.0 (fixed)	0.8 (fixed)	none	none	none	none
91	*Pmin*/*Pmax* fixed, no tails	**1635.3 (1545.3-1715.8)**	286.7 (171.7-473.5)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
92	*Pmin*/*Pmax* fixed, no tails	1727.4 (1662-1770.1)	114.7 (45.9-294.8)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
93	*Pmin*/*Pmax* observed, no tails	1558.2 (1102.4-1856.8)	**2401.2 (1368.1-3251.8)**	0.1 (fixed)	1.0 (fixed)	none	none	none	none
94	*Pmin*/*Pmax* fixed, no tails	1562.2 (1243.7-1749.8)	**1418.9 (914.5-2539.3)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none
95	*Pmin*/*Pmax* fixed, no tails	1683.2 (1594.4-1754.6)	267.8 (145.1-461)	0.0 (fixed)	1.0 (fixed)	none	none	none	none
96	*Pmin*/*Pmax* estimated, no tails	1682.9 (1460.1-1746.3)	**140.3 (0.1-508.8)**	0.1 (0.0-0.2)	1.0 (0.8-1.0)	none	none	none	none
97	*Pmin*/*Pmax* estimated, no tails	1974.4 (1903.7-2061)	**124.2 (0-371.9)**	0.1 (0.0-0.2)	0.6 (0.5-0.8)	none	none	none	none

98	*Pmin*/*Pmax* fixed, right tail	1705.9 (1604.9-1756.9)	166.9 (18.8-364.7)	0.0 (fixed)	1.0 (fixed)	none	none	157.3 (0.1-445.2)	0.1 (0.0-1.0)
99	*Pmin*/*Pmax* observed, no tails	**1630.7 (1514.8-1725.5)**	**266.8 (108.6-521.5)**	0.0 (fixed)	0.9 (fixed)	none	none	none	none
100	*Pmin*/*Pmax* fixed, right tail	1720.9 (1572.7-1904.3)	**360.2 (162-933.3)**	0.0 (fixed)	1.0 (fixed)	none	none	0.8 (0.1-453.9)	0.4 (0.0-1.0)
101	*Pmin*/*Pmax* observed, no tails	**2333.2 (2171.4-2607.3)**	**980.5 (584.2-1842.8)**	0.1 (fixed)	1.0 (fixed)	none	none	none	none
102	*Pmin*/*Pmax* fixed, no tails	**1617.9 (1477.9-1729.9)**	**637.9 (432.5-1007.3)**	0.0 (fixed)	1.0 (fixed)	none	none	none	none

CHAPTER 3

EXPERIMENTAL EVIDENCE THAT EXTRA-PAIR MATING DRIVES ASYMMETRICAL INTROGRESSION OF A SEXUAL SIGNAL

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Abstract

Theory suggests that traits under positive selection may introgress asymmetrically across a hybrid zone, potentially driven by sexual selection. Two subspecies of the red-backed fairy-wren (*Malurus melanocephalus*) differ primarily in a sexual signal used in mate choice – red vs. orange male back plumage color – but phylogeographic analyses suggest asymmetrical introgression of red plumage into the genetic background of the orange subspecies. I hypothesized that this asymmetrical introgression may be facilitated by sexual selection if red males have a mating advantage over orange males. I tested this hypothesis with correlational data and a plumage manipulation experiment where I reddened the back plumage of a subset of orange males to mimic males of the red subspecies. There was no correlational evidence of a mating advantage to naturally redder males in this population. Experimentally reddened males sired a similar amount of within-pair young and lost paternity at the same rate as orange males, but they sired significantly more extra-pair young, leading to substantially higher total reproductive success. Thus I conclude that sexual selection via extra-pair mating is a likely mechanism responsible for the asymmetrical introgression of plumage color in this system, and is potentially driven by a sensory bias for the red plumage signal.

Introduction

Sexual selection is a powerful evolutionary force that can restrict gene flow between diverging taxa upon secondary contact (Irwin and Price 1999; Questiau 1999; Servedio et al. 2007). However, sexual selection might also facilitate gene flow between taxa when reproductive isolation is incomplete and there is little or no cost to hybridization. When hybridizing taxa are at an intermediate stage of divergence, reproductive isolation is often predicted to be asymmetrical (Kaneshiro 1980; Arnold et al. 1996), and may lead to asymmetrical introgression of traits under positive selection from one population to another. This idea has received theoretical support (Anderson and Stebbins 1954; Barton 1979; 2001), but empirical evidence is limited (Rohwer and Wood 1998; Arnold 2004). One of the best examples comes from golden- and white-collared manakins (*Manacus* spp.), where the asymmetrical introgression of a secondary sexual trait, male throat plumage color (Parsons et al. 1993; Brumfield et al. 2001), has been driven by sexual selection (Stein and Uy 2006; Uy and Stein 2007). However, the extent to which this phenomenon is more widespread is unclear, and the underlying mechanisms driving such patterns are seldom investigated (but see Pearson and Rohwer 2000; Bronson et al. 2003).

The red-backed fairy-wren (*Malurus melanocephalus*) is a small, insectivorous Australian passerine that appears to exhibit asymmetrical introgression of a secondary sexual trait (Chapter 2). There are two recognized subspecies that differ primarily in a sexual signal, male nuptial plumage color (Karubian 2002; Webster et al. 2008; Karubian et al. 2009): the red-backed *M. m. cruentatus* and the orange-backed *M. m. melanocephalus* (hereafter referred to as "red" and "orange", Figure 3.1). The subspecies are genetically differentiated at multiple nuclear and mitochondrial loci across the Carpentarian Barrier, with an estimated divergence time of 270,000 years ago (Lee and Edwards 2008). The Carpentarian Barrier is a prominent arid and sparsely vegetated zoogeographic barrier for many other Australian taxa, and is thought to have been a particularly impermeable barrier during the Pleistocene (Cracraft 1986; Ford 1987; Jennings and Edwards 2005). The current continuous distribution of the red-backed fairy-wren across the Carpentarian Barrier likely resulted from secondary contact as the species re-colonized

this area after refugial isolation in coastal northern and southeastern Australia (Cracraft 1986; Lee and Edwards 2008). Males of the two subspecies have different song characteristics that are also divergent across this barrier, and males respond most strongly to their own subspecies' song (Greig and Webster 2013). In stark contrast, the contact zone between plumage types occurs several hundred kilometers to the east of the Carpentarian Barrier, well within the genetic background of the orange *M. m. melanocephalus* subspecies (Chapters 1 and 2; Baldassarre et al. 2013, Figure 3.1).

Plumage color in the red-backed fairy-wren is carotenoid-based (Rowe and McGraw 2008) and because birds must ingest carotenoids, variation in the amount of carotenoids used in signals is often thought to be strictly related to underlying environmental variation (Hill 1992). However, in the red-backed fairy-wren, variation in plumage color is not well explained by environmental variation alone, but instead exhibits a pattern of isolation by distance, suggesting a significant genetic component (Chapter 1; Baldassarre et al. 2013). In addition, there is a particularly high rate of change in plumage color across the eastern contact zone, and no significant change across the Carpentarian Barrier (Chapter 1; Baldassarre et al. 2013). There is no significant genetic structure across the contact zone between plumage types (Chapter 2; Lee and Edwards 2008), and no other morphological traits are divergent across the plumage contact zone (Chapter 2; Baldassarre et al. 2013). Taken together, these patterns are most consistent with the hypothesis of asymmetrical introgression of a single trait – red plumage – across the hybrid zone following secondary contact. Previous studies have shown that plumage color is subject to sexual selection, as females prefer males in bright nuptial plumage to cryptically-colored males (Karubian 2002; Webster et al. 2008; Karubian et al. 2009). These studies suggest that females assess the back plumage of potential mates, so I hypothesized that sexual selection may be the force driving the introgression of red plumage color across the hybrid zone.

I tested the prediction that red males have a mating advantage over orange males with correlational analyses and a plumage manipulation experiment in which I reddened the back plumage of males in a population of the orange *M. m. melanocephalus* subspecies to mimic

males of the red *M. m. cruentatus* subspecies. For both approaches, I predicted that males with redder (*cruentatus*-like) plumage would have higher reproductive success than more orange (*melanocephalus*-like) males. I further predicted that the mating advantage of both naturally redder and experimentally reddened males would be due in large part to siring more extra-pair young and being cuckolded (losing paternity) less than orange males. Because red-backed fairy-wrens exhibit high rates of extra-pair paternity and high variance in extra-pair mating (Karubian 2002; Webster et al. 2008), this component of their reproductive success is thought to be the main factor contributing to the opportunity for sexual selection (Webster et al. 1995) in this species, as it is for other fairy-wren species (Webster et al. 2007).

Methods

Population monitoring and plumage color quantification

This study was conducted during two breeding seasons in an allopatric population of the orange *M. m. melanocephalus* subspecies on Lake Samsonvale (27°16'S, 152°41'E), 30 km NW of Brisbane, Queensland, Australia, and about 1,200 km south of the plumage contact zone (Figure 3.1). I extensively monitored the population for one breeding season (from September 2010 to January 2011) to collect data for my correlational analyses. During this period, I captured, color-banded, and took blood samples from adults, and found all nests in order to band and collect blood samples from nestlings. I collected standard morphometric measurements (e.g., weight, tarsus length, tail length) from all captured birds, and also collected feather samples (6-12 feathers from the orange back patch) from all males in nuptial plumage. Daily monitoring of individuals allowed me to quantify the date at which males completed their nuptial molt, which is an important indicator of age and reproductive success in a congener, the superb fairy-wren (*Malurus cyaneus*, Cockburn et al. 2008), and may be in the red-backed fairy-wren as well (Karubian 2002). I was able to classify adult birds into two age classes – second-year and after-second-year – based on degree of skull ossification (Lindsay et al. 2009), which was important because in a different population of red-backed fairy-wrens, older males tend to foray off their

territories – likely seeking extra-pair copulations – more often than do younger males (unpublished data).

I quantified plumage color (i.e., color of the orange back patch of each male) using reflectance spectrophotometry of the collected feathers and a mathematical model of the avian visual system as described in Chapter 1 and Baldassarre et al. (2013). Briefly, I analyzed reflectance curves with the program TetraColorSpace (Stoddard and Prum 2008) using the average avian VS cone-type retina and idealized illumination. I then extracted the angle of the color vector (hue), achieved chroma (saturation), and normalized brilliance (brightness) for each feather sample. TetraColorSpace produces two values for hue that describe the angle of the color vector: θ (theta) and φ (phi). I focused on theta for my quantification of hue because it best captures the variation from orange to red that characterizes the subspecies (Chapter 1; Baldassarre et al. 2013), but the results for phi were qualitatively similar (see below).

Plumage manipulation

I conducted the plumage manipulation experiment during the subsequent breeding season (September 2011 to January 2012). My experimental design consisted of three groups of 13 males each: "reddened", "sham", and "control". I manipulated the plumage of reddened males using a non-toxic permanent art marker (Prismacolor Carmine Red®) and applied a colorless marker (Prismacolor Colorless Blender®) to the sham males. I included the sham group in the experimental design to control for potential effects of marker application. Control males were captured and handled in the same way, but without any plumage alteration.

One way to examine variation in plumage color between subspecies and compare this variation to color resulting from the plumage manipulation is to compare the average reflectance curves (Figure 3.2). The major difference between *M. m. melanocephalus* (the eastern, orange subspecies) and *M. m. cruentatus* (the western, red subspecies) is a shift in the dominant reflectance into longer wavelengths, corresponding to a shift in hue from orange to red. The average reflectance curve of sham treated males very closely mimicked that of naturally varying

orange males, and the average reflectance curve of reddened males very closely mimicked that of naturally varying red males of the non-local *M. m. cruentatus* subspecies (Figure 3.2). Furthermore, when I plotted the color of feather samples from control *M. m. cruentatus* males, sham treated males, naturally red *M. m. melanocephalus*, and experimentally reddened males as clouds of points in tetrahedral color space using the R package *pavo* (Maia et al. 2013), I found two separate clusters representing "orange" and "red" color, characterized by different color vector angles (Figure 3.3). The "orange" cluster was comprised of control and sham treated males from the study population, and the "red" cluster was comprised of naturally varying *M. m. cruentatus* males and experimentally reddened males.

These comparisons were corroborated by comparing hue, saturation, and brightness among groups as quantified by TetraColorSpace (Figures 3.4a and 3.5). There is relatively little variation between the subspecies in saturation (measured by achieved chroma, the relative length of the color vector compared to the maximum) and brightness (measured by normalized brilliance, the standardized total reflectance between 300 and 700 nm), and the manipulations did not affect these color variables (see below). While I focused on theta as my hue metric (Figure 3.4a) theta and phi are correlated, and the results for phi were qualitatively similar to those for theta (compare Figures 3.4a and 3.5). To analyze the effect of the manipulations on plumage color, I used paired sample *t*-tests to compare the color before and after manipulation. The colorless marker used in the sham manipulation did not significantly alter plumage hue, saturation, or brightness (all t between -1.4 and -0.5, df = 12, all $p > 0.1$). The red marker did not alter saturation ($t = -1.57$, df = 12, $p = 0.15$), or brightness ($t = 1.85$, df = 12, $p = 0.09$), but it significantly reddened the hue of males ($t = -19.8$, df = 12, $p < 0.001$, Figure 3.4), and these birds obtained a red hue that was indistinguishable from the natural hue of the red *M. m. cruentatus* subspecies, which was the desired effect (*t*-test $t = 1.5$, df = 22.9, $p = 0.16$, Figure 3.4).

At the beginning of the breeding season – after males had completed their pre-nuptial molt, secured a territory, and formed social pairs, but before I observed any nesting behavior – I arbitrarily assigned 13 males to the reddened group, maximizing the distance between territories.

This spatial arrangement minimized the potential for reddened males to compete with each other, and for females to choose between multiple reddened males. I then arbitrarily assigned 13 males to the sham group and 13 males to the control group. Prior to manipulation, experimental groups did not differ in plumage color as measured by hue (Figure 3.4a), saturation, or brightness (all ANOVA $F_{2,36} < 2$, all $p > 0.1$). Experimental groups were also similar in other potentially important characteristics. Although it was not possible to obtain a precise age of each bird, all experimental males were after-second-year birds (i.e., no males were inexperienced breeders), and my arbitrary assignment of males to experimental groups likely minimized any major hidden age effects. Also, there were no differences between experimental groups in date of nuptial molt completion (ANOVA $F_{2,36} = 0.88$, $p = 0.42$), tail length ($F_{2,36} = 0.48$, $p = 0.63$), weight ($F_{2,36} = 0.05$, $p = 0.95$), or body condition measured as the residual of a regression of weight on tarsus length ($F_{2,36} = 0.02$, $p = 0.99$). Pilot studies indicated that the red marker began to fade after a month, so I re-captured reddened males at monthly intervals and re-applied the marker. All sham and control males were re-captured at least once, and the colorless marker was re-applied to the sham males. After initial manipulation, I released all experimental males back into the population and monitored all subsequent nesting attempts.

Genetic paternity analyses

I extracted DNA from embryos and blood samples using the Omega Bio-Tek EZ-96 Total DNA/RNA Isolation Kit®. I then genotyped all individuals at seven highly polymorphic microsatellite loci isolated from different bird species (Table 3.1). For PCR reactions, I added 1 µl of extracted DNA to a mixture including 1.3 µl of 25 mM $MgCl_2$, 1 µl of 10x PCR buffer (Sigma), 0.2 µl of 10 mM dNTPs, 0.12 µl of each 10 mM PCR primer (one of each pair labeled with a fluorescent dye), 0.1 µl of 2.5U/µl *Taq* polymerase (Sigma Jumpstart®), and DNA water up to a total volume of 10 µl per well. I used the following PCR conditions: following an initial 3 min denaturation at 94°C, the reaction mix went through 35 cycles of 94°C for 30 sec, X°C for 30 sec, and 72°C for 1 min, ending with a cycle at 72°C for 5 min where X was the optimized

annealing temperature or a touchdown procedure from 58-56°C (Table 3.1). After PCR, I created two mixtures for genotyping, consisting of 1 µl PCR products from either three or four loci, each labeled with a different dye (6FAM, PET, VIC, or NED), combined with 11.9 µl formamide and 0.1 µl size standard (GeneScan-500 LIZ®) for each well. PCR products were separated on an ABI Prism 3730® automated sequencer, and alleles were scored using the program GeneMapper (Applied Biosystems) and verified by eye.

To assign the paternity of each offspring, I assumed the breeding female observed incubating and/or feeding nestlings was the genetic mother of all offspring in that nest, as has been confirmed in previous analyses of genetic parentage in this species (Webster et al. 2008). I was able to further test the validity of this assumption by examining allele mismatches between the mother and offspring in her nest. If a given offspring were the result of brood parasitism, it should mismatch with the mother at the nest at multiple loci. I never observed greater than two mismatches between offspring and assumed mothers, and attributed these to the presence of null alleles or scoring errors. I assigned paternity using the program CERVUS 3.0 (Kalinowski et al. 2007), which determines which male in the population has the highest likelihood of siring a given offspring. CERVUS calculates a log likelihood score (LOD) for each male accounting for offspring genotype, maternal genotype, and genotype scoring errors (e.g., due to null alleles). For each assignment, I used a "total evidence" (Prodöhl et al. 1998) approach to validate the CERVUS assignment. In most cases, I accepted the CERVUS assignment if the male chosen had 0 or 1 mismatch with the nestling, but I rejected the CERVUS assignment and assigned paternity to a male with a lower LOD score under three circumstances: 1) if both males had similar LOD scores but the lower ranked male had fewer mismatches, 2) if both males had a single mismatch but the lower ranked male's mismatch could be explained by a null allele, and 3) if the males had the same low number of mismatches and similar LOD scores, but independent evidence suggested that the lower ranked male was a more likely sire (after Webster et al. 2004). Independent evidence that I took into account included whether a candidate male was the social father, sired other offspring in the nest, exhibited a mismatch consistent with a scoring error, or

was highly unlikely to have copulated with the female based on an unreasonably large distance between territories. Using this additional evidence likely improved the reliability of several assignments, but was unlikely to affect overall patterns because I accepted the CERVUS-assigned male in the majority of cases (see below).

When combined, the microsatellite loci were highly polymorphic and informative for paternity analysis (mean number of alleles per locus = 11.43, mean expected heterozygosity = 0.689, Table 3.1). Allele frequencies did not deviate from Hardy-Weinberg expectations, but two loci (*Mcy2* and *Smm7*) had an estimated null allele frequency greater than 0.05, which was taken into account during subsequent paternity assignments. The average probability of excluding a randomly chosen male as the father was high, with a combined probability of exclusion of 0.998. The correlational dataset consisted of 89 genotyped offspring. Of these, I was able to assign paternity to 77 (86.5%), and accepted the CERVUS-assigned male 98.7% of the time (I only used independent evidence to assign the paternity of one offspring). The plumage manipulation dataset consisted of 185 genotyped offspring, including 143 nestlings and 42 eggs. Of these, I was able to assign paternity to 178 (96.2%), and accepted the CERVUS-assigned male 85% of the time. Of the 48 extra-pair young that were assigned to reddened males, all were clear-cut cases of extra-pair paternity where the social male mismatched at three or more loci. Furthermore, of these 48 cases, I only used independent evidence to overrule the CERVUS assignment and assign paternity to a reddened male in six cases. The offspring to which I could not assign paternity were sampled from nests on the edge of the study site and although I could confirm that they were extra-pair young, they were likely sired by un-captured males that held territories adjacent to the site. This occurs relatively rarely because the majority of the study site is bordered by water. Because the experimental males were embedded in a larger population of non-experimental birds, the vast majority of extra-pair young were sired in the nests of non-experimental males. This means that an increase in extra-pair paternity for one experimental group did not necessarily lead to a decrease in within-pair paternity for other groups. The paternity analyses revealed a high overall level of extra-pair paternity in the population, with

64.1% and 57.3% of all offspring resulting from extra-pair copulations, and 73.2 % and 71.3% of all broods containing at least one extra-pair young in 2011 and 2012 respectively.

Using these paternity results, for each male I calculated within-pair reproductive success (total number of offspring sired in the nests of a male's social mate), extra-pair reproductive success (total number of offspring sired in the nests of other males), total reproductive success (total number of offspring sired in the population), probability of being cuckolded (a male having at least one offspring in his own nest sired by another male), and cuckoldry rate (proportion of offspring in a male's own nests that were sired by another male).

Statistical analyses

For non-normally distributed response variables I analyzed the data using either Poisson or binomial distributions, or non-parametric mean comparisons. Using the correlational data, I analyzed the effect of plumage color on reproductive success seven different ways. First, I fit four generalized linear models with plumage hue as the predictor variable and either number of within-pair young, number of extra-pair young, number of extra-pair mates, or total reproductive success as response variables. For these models I used a Poisson distribution with a log link function. I also examined a male's general success at achieving extra-pair paternity by scoring whether or not he sired at least one extra-pair young (either 0 or 1), and whether or not he was cuckolded by his social mate (either 0 or 1). I used these metrics as response variables in generalized linear models with plumage hue as the predictor variable with a binomial distribution and a logit link function. Finally, I conducted a pairwise analysis of the plumage hue of a cuckolded male compared to the plumage hue of the male that cuckolded him using a paired *t*-test. I analyzed the results of the plumage manipulation experiment using Kruskal-Wallis mean comparisons of number of within-pair, extra-pair, and total offspring sired between experimental groups, and then conducted pairwise comparisons using Wilcoxon Rank-Sum tests. All statistical analyses were performed using R version 2.15 (R Core Team 2012).

Results

Natural plumage hue and reproductive success

I found no effect of naturally varying plumage hue on reproductive success. Natural plumage hue had no significant effect on number of within-pair young, number of extra-pair young, number of extra-pair mates, total reproductive success, probability of siring at least one extra-pair young, or probability of being cuckolded (Table 3.2). In addition, there was no significant difference in plumage hue between males that lost paternity and the males that cuckolded them (paired *t*-test, $t = -0.17$, df = 19, $p = 0.87$).

Effect of plumage manipulation on reproductive success

Plumage manipulation significantly affected male extra-pair reproductive success. There was no difference between experimental groups in number of within-pair young (Kruskal-Wallis $H = 3.7$, df = 2, $p = 0.16$, Figure 3.6a), but there was a significant difference in number of extra-pair young ($H = 8.72$, df = 2, $p = 0.01$, Figure 3.6b), which resulted in a significant difference in total reproductive success ($H = 7.27$, df = 2, $p = 0.03$, Figure 3.6c). In pair-wise comparisons between groups, control and sham males sired a similar number of extra-pair young (Wilcoxon Rank-Sum $W = 82$, $n_{1,2} = 13$, $p = 0.91$), but reddened males sired significantly more extra-pair young than both control ($W = 31$, $n_{1,2} = 13$, $p = 0.003$) and sham males ($W = 30$, $n_{1,2} = 13$, $p = 0.005$). Similarly, control and sham males had equal total reproductive success ($W = 85.5$, $n_{1,2} = 13$, $p = 0.98$), but reddened males had higher total reproductive success than both control ($W = 37.5$, $n_{1,2} = 13$, $p = 0.02$) and sham males ($W = 35.5$, $n_{1,2} = 13$, $p = 0.01$). Plumage manipulation had no effect on the proportion of males cuckolded (Kruskal-Wallis $H = 2.91$, df = 2, $p = 0.23$) or cuckoldry rate ($H = 4.62$, df = 2, $p = 0.1$). As a final way to compare effect of hue on reproductive success in the naturally-varying population and following the plumage manipulation, I plotted naturally varying hue in 2011 and population-wide hue in 2012, including reddened males, on the same x-axis scale, with number of extra-pair young on the y-axis (Figure 3.7). In 2011, with the limited amount of natural variation in hue, I found no relationship

between hue and number of extra-pair young, as presented above (Table 3.2). In 2012, when I increased the variation in hue by introducing reddened males that mimic the naturally red *M. m. cruentatus* subspecies, I found a significant positive association (GLM: $N = 59$, estimate = 6.6, SE = 1.5, $t = 4.35$, $p < 0.001$).

Discussion

Asymmetrical introgression via extra-pair mating

I found support for the hypothesis that sexual selection via extra-pair mating has driven the asymmetrical introgression of red back plumage from the *M. m. cruentatus* subspecies into the genetic background of the orange *M. m. melanocephalus* subspecies described in Chapter 2. Experimentally reddening the plumage of a subset of males in an orange population resulted in a higher number of extra-pair young, which led to higher total reproductive success than control and sham males (Figure 3.6). These reddened males, however, sired a similar number of within-pair young as control and sham males, suggesting that the increase in total reproductive success was due exclusively to an extra-pair mating advantage. Other populations of red-backed fairy-wrens are known to exhibit low variance in within-pair paternity and high rates of extra-pair paternity (Karubian 2002; Webster et al. 2008), and this study revealed a similarly high rate in the experimental population. Because of this, extra-pair paternity is thought to be the main component contributing to sexual selection in this species (see also Webster et al. 2007), and the evolutionary force driving asymmetrical introgression of this sexual signal.

Behaviorally, there is evidence that females have the opportunity to assess potential extra-pair mates. Males foraying onto a neighboring male's territory exhibit "petal carrying" in which they display flower petals to potential extra-pair females (Rowley and Russell 1997; Karubian and Alvarado 2003). Foraying males also display their back plumage to females in a stereotyped "puff-back display" (Rowley and Russell 1997). I suggest that it is during these displays that females assess the back plumage of males, and that they actively prefer red males to orange males, leading to the observed difference in extra-pair reproductive success. However,

extra-pair copulations are rarely observed, and I did not quantify any behavioral differences between experimental groups.

Although reddened males sired more extra-pair young than control and sham males, they were cuckolded just as often, resulting in similar levels of within-pair paternity. These results suggest that females use different cues or signals to assess the quality of a social mate such as territory quality, age, parental ability, or genetic similarity (Varian-Ramos and Webster 2012) among other possibilities. Several studies have shown that different male traits contribute to within- and extra-pair reproductive success (Bitton et al. 2007; Lehtonen et al. 2009; O'Brien and Dawson 2010). This may be due in part to the different contexts in which females are likely to assess these two types of mates. In the red-backed fairy-wren, females likely have ample opportunity to evaluate within-pair mates, but may be under significant temporal constraint when assessing potential extra-pair mates due to mate guarding and the brevity of territorial intrusions (unpublished data). Under these circumstances, plumage color may be a signal that is easy for a female to identify and assess quickly. Alternatively, females may have chosen social mates on the basis of plumage color but not re-assessed them post-manipulation, although a study on barn swallows (*Hirundo rustica*) suggests that some bird species are capable of dynamic evaluation and paternity allocation (Safran et al. 2005). However, plumage color is likely not a consistent and reliable signal during pair formation, as this typically occurs before males have completed their pre-nuptial molt (D. Baldassarre, pers. obs.). Finally, females may be too constrained by the lack of vacant territories at the beginning of the season to exhibit a preference for male phenotype.

Sensory bias in females

I found no support for the hypothesis that naturally redder males have higher reproductive success than more orange males in this population (Table 3.2). Because the study population is located in an allopatric area of the orange subspecies, there is little variation in plumage color compared to populations within the plumage contact zone (Brisbane hue SD = 0.02 vs. 0.04 in

contact zone, see Chapter 1; Baldassarre et al. 2013). Only when experimentally manipulating the plumage of males outside the natural range of hue found in this population did I detect an effect (Figure 3.7). This suggests that *M. m. melanocephalus* females may have a latent preference (i.e., sensory bias, Ryan 1990) for redder males that would only become apparent in an area where the two plumage types co-occur. This may be the mechanism acting at the leading edge of the plumage contact zone, causing it to move asymmetrically. However, whether females prefer red because it is a novel trait (Burley and Symanski 1998) or because of a specific bias toward red is unclear. I suggest that the observed asymmetrical nature of the introgression is consistent with females of both subspecies preferring red males, although a similar study would need to be conducted in a population of the red *M. m. cruentatus* subspecies to thoroughly test this hypothesis (I was unable to develop a reliable method to make the plumage of naturally red males more orange). In addition, red males may have an advantage over orange males if the red signal is more easily detected and is more conspicuous against the visual background (i.e., sensory drive, Endler and Basolo 1998). This is the mechanism invoked to explain the asymmetrical introgression of golden plumage in the hybrid zone between golden- and white-collared manakins, where golden plumage is more visually conspicuous than white (Uy and Stein 2007).

Another possibility is that the introgression is driven by competitive interactions between males. In the hybrid zone between black-capped chickadees (*Poecile atricapillus*) and Carolina chickadees (*P. carolinensis*) females of both species prefer Carolina chickadee males as extra-pair mates because they prefer dominant males, and Carolina chickadees are dominant over black-capped chickadees (Bronson et al. 2003). I did not quantify any behavioral differences between experimental groups in this study, but a mount presentation experiment to determine if territorial males differ in their aggressive responses to red vs. orange mounts is the subject of Chapter 4. Finally, red plumage may be an honest indicator of quality in this system, but I suggest that the lack of selection on naturally varying plumage hue is inconsistent with this hypothesis, and I did not find any association between male condition and hue (unpublished

data).

Implications for gene flow

Theory suggests that strong positive selection on an allele in the alternative environment and genetic background is necessary for introgression across a hybrid zone (Barton 1979; 2001). My experimental results suggest that extra-pair mating may provide the mechanism by which this can occur, thereby facilitating gene flow and introgression of an advantageous trait from one subspecies to the other. Specifically, at the plumage contact zone where red and orange males co-occur, *M. m. melanocephalus* females may choose orange males as social mates, but prefer red *M. m. cruentatus*-like males as extra-pair mates, leading to higher total reproductive success for red males, and introgression of alleles for red back plumage into the genetic background of the orange subspecies. This is precisely the pattern revealed by geographic cline analyses of the hybrid zone (Chapter 2), as clines for many unlinked SNP loci are centered at the Carpentarian Barrier, while the plumage cline is displaced 390 km to the east. Because this gene flow is driven by extra-pair mating, these results would hold even if the subspecies pair assortatively within the genetic hybrid zone. Assortative pairing may be mediated by song in this species, as males of the two subspecies respond most strongly to the song of their own subspecies, and variation in song is coincident with genetic variation, suggesting it has been resistant to introgression (Greig and Webster 2013). My findings support recent theory suggesting that alternative mating tactics such as extra-pair mating can erode species boundaries and facilitate introgression even if taxa pair assortatively (Hartman et al. 2011).

Conclusions

In summary, I found support for the hypothesis that asymmetrical introgression of a sexual signal – red back plumage color – is facilitated by sexual selection in the red-backed fairy-wren. This introgression is likely mediated solely by extra-pair mating behavior, as I did not detect an effect of plumage manipulation on within-pair reproductive success. Because I did not find any effect

of naturally varying plumage color on reproductive success in the orange population, this likely represents a latent preference in females. This study highlights the importance of considering receiver response to diverging sexual signals during speciation. In taxa that are at an intermediate stage of divergence, receiver response may not have diverged in tandem with signals. Upon secondary contact, asymmetry in receiver response may promote the spread of an advantageous sexual trait. This may represent a potent conduit for asymmetrical introgression in other species with high levels of extra-pair paternity.

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Table 3.1 Characteristics of microsatellite loci used for paternity analysis. Probability of exclusion is the probability of excluding a randomly selected, unrelated male as the sire given the genotype of the offspring and mother. References for primers are as follows: all *Mcy* loci from Double et al. (2007), *Msp6* from Webster et al. (2004), *Ase9* from Richardson et al. (2000), and *Smm7* from Maguire et al. (2006).

Locus	Annealing temp. (°C)	# alleles	Expected het. (*He*)	Observed het. (*Ho*)	Prob. of exclusion	Null freq.
Mcy1	55-58	9	0.717	0.737	0.509	-0.021
Mcy2	61	4	0.088	0.076	0.044	0.071
Mcy4	55-58	12	0.822	0.761	0.66	0.035
Mcy7	61	17	0.806	0.820	0.647	0.014
Msp6	55-58	8	0.698	0.686	0.463	0.001
Ase9	61	14	0.788	0.766	0.62	0.015
Smm7	61	16	0.810	0.810	0.802	0.053

Table 3.2 Plumage hue and reproductive success in 2011. Results of six generalized linear models of the effect of naturally varying plumage hue on various measures of reproductive success.

Model	*N*	Estimate	SE	*t*	*p*
Number of within-pair young	57	-10.589	9.683	-1.092	0.275
Number of extra-pair young	57	-5.59	7.693	-0.727	0.467
Number of extra-pair mates	57	-8.407	10.444	-0.805	0.421
Total reproductive success	57	-7.484	6.023	-1.243	0.214
Sired ≥ extra-pair young	57	-0.542	14.913	-0.036	0.971
Cuckolded	57	0.875	21.311	0.041	0.967

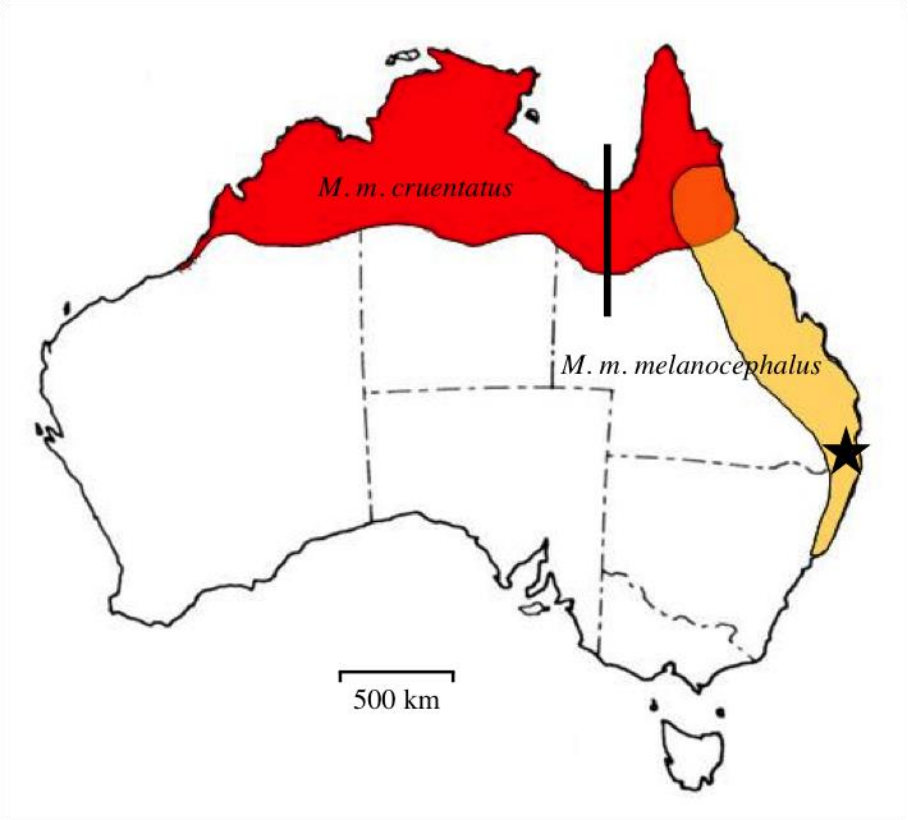

Figure 3.1 The species range of the red-backed fairy-wren showing the distribution of the two different colored subspecies. The red *M. m. cruentatus* occurs in the north, and the orange *M. m. melanocephalus* occurs in the east. The intermediately colored area in the northeast represents the approximate contact zone between plumage types. The vertical line represents the genetic boundary between subspecies, the Carpentarian Barrier. The population where the study was conducted is marked with a star.

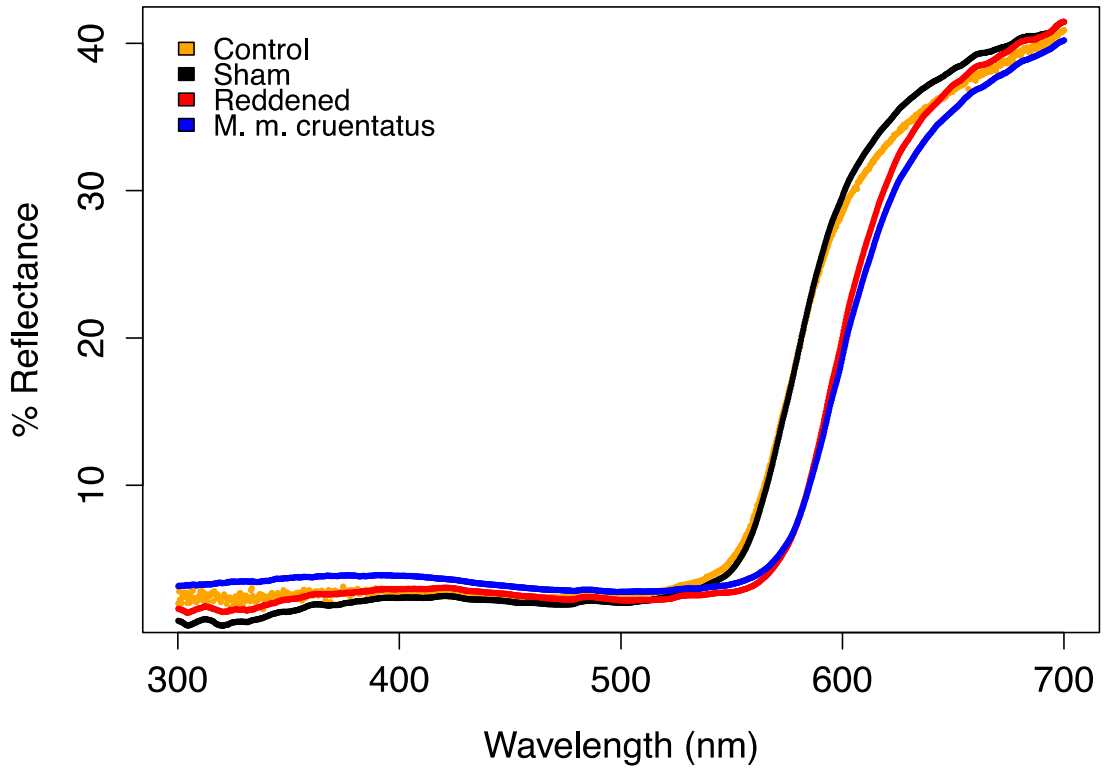

Figure 3.2 Typical reflectance curves for the orange-red back plumage of red-backed fairy-wren males. Each curve represents the average reflectance curve for control males in the study population (orange), sham treated males (black), naturally red *M. m. cruentatus* males (blue), and experimentally reddened males (red).

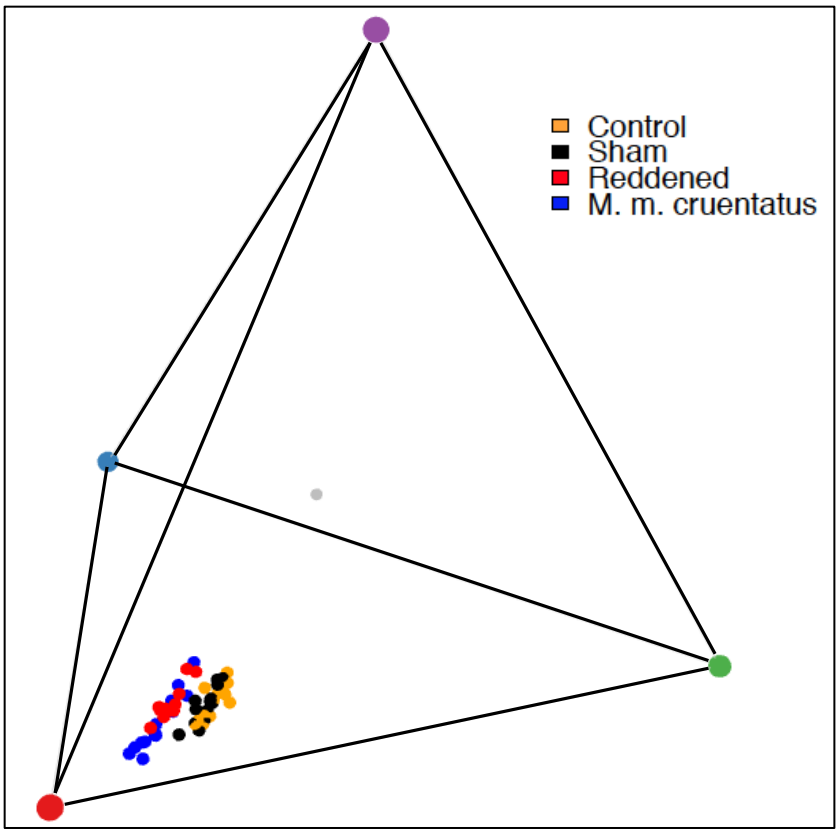

Figure 3.3 How various red-backed fairy-wren plumage colors are represented in the tetrahedral color space. Each color falls as a point in the color space based on the relative stimulation of the four cone channels (purple = violet, blue = short, green = medium, red = long). The grey dot in the middle represents the achromatic origin.

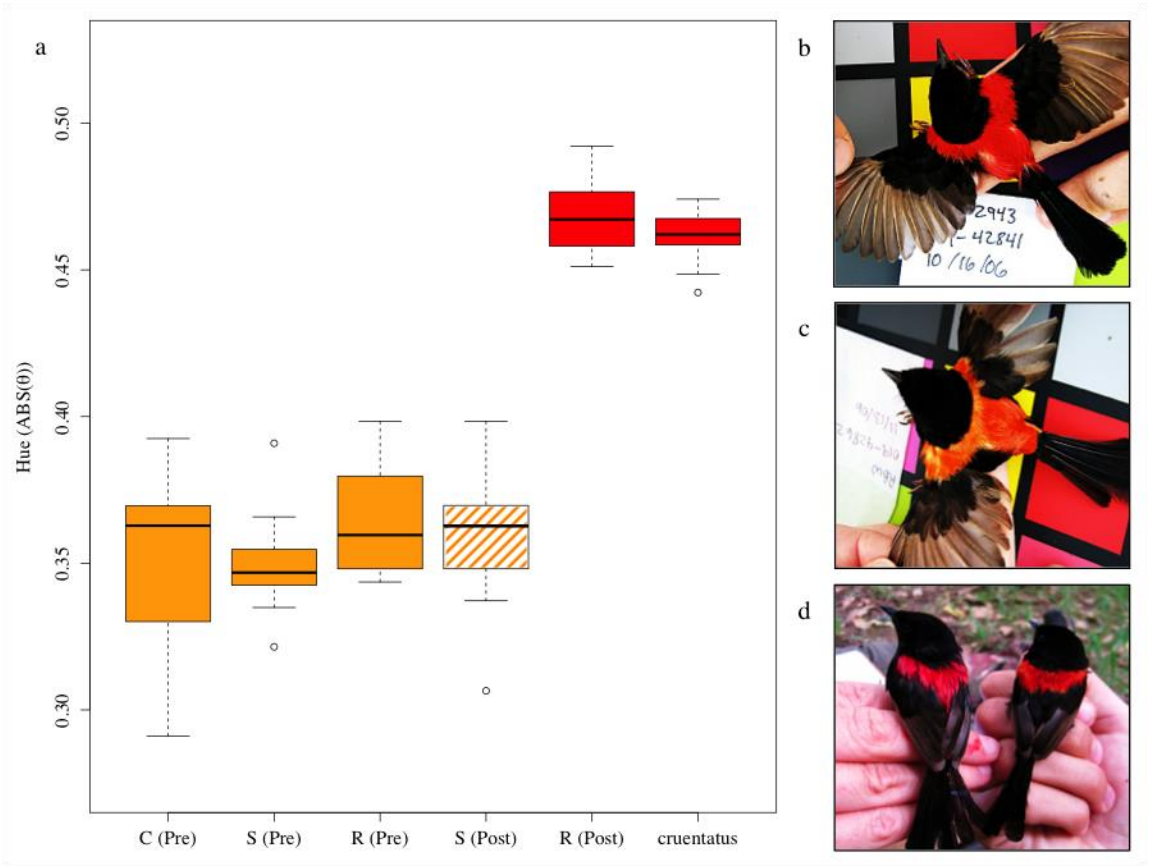

Figure 3.4 The effect of the plumage manipulation on hue as measured by theta. Boxplots show comparison of plumage hue between experimental groups (C = control, S = sham, R = reddened) pre- and post-manipulation, and comparison of experimentally reddened males to the natural hue of the red *M. m. cruentatus* subspecies (a). Values for plumage hue have been converted to absolute values for visualization purposes. The panels on the right show a red *M. m. cruentatus* male (b), an orange *M. m. melanocephalus* male (c), and a photo comparing an experimentally reddened male to a control male (d).

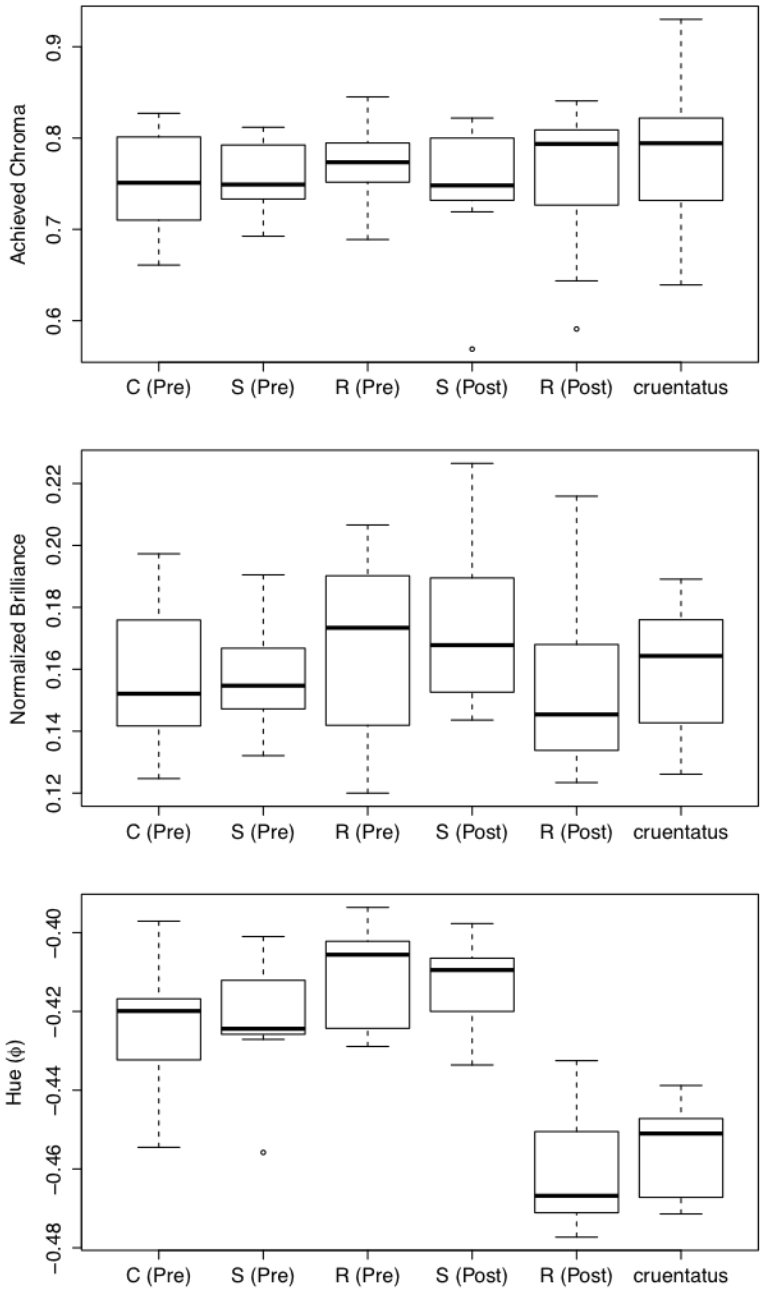

Figure 3.5 Description of variation in other color metrics. Boxplots show comparison between experimental groups (C = control, S = sham, R = reddened) pre- and post-manipulation, and the naturally red *M. m. cruentatus* subspecies in achieved chroma (i.e., saturation), normalized brilliance (i.e., brightness), and hue as measured by phi (φ).

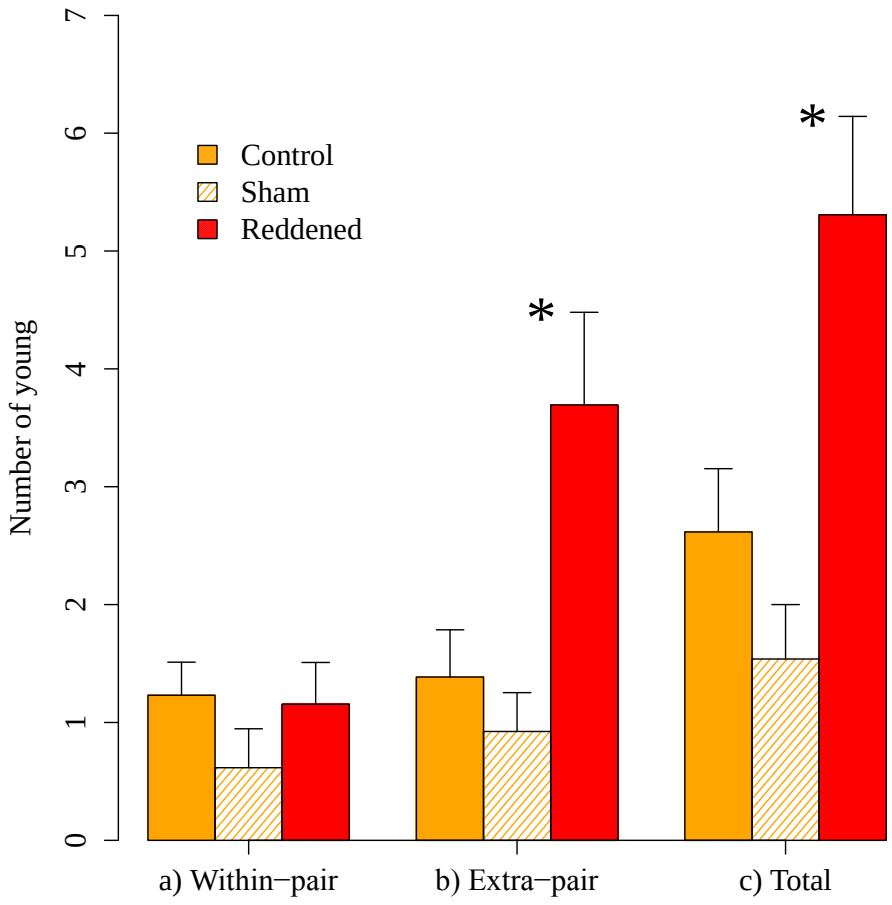

Figure 3.6 The effects of the plumage manipulation on reproductive success. Reproductive success is divided into number of within-pair young (a), number of extra-pair young (b), and total number of young (c). Error bars represent standard errors, and asterisks separate values that are significantly different.

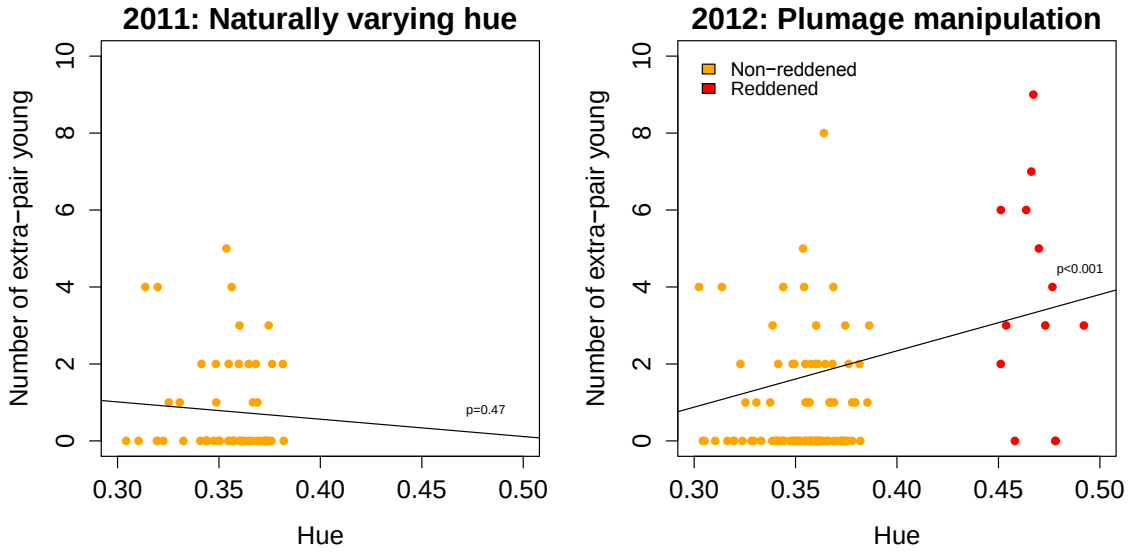

Figure 3.7 How naturally varying and manipulated plumage hue affected extra-pair mating success. The two plots compare the effect of plumage hue on number of extra-pair young in 2011 (left panel) when only naturally varying orange males were present in the population, and in 2012 (right panel) when experimentally reddened males were introduced.

CHAPTER 4

DISCRIMINATION BASED ON SONG BUT NOT PLUMAGE IN A SUBSPECIES PAIR WITH DIFFERENTIAL INTROGRESSION OF SOCIAL SIGNALS

Abstract

Socially selected signals such as plumage and song in birds often diverge in allopatry or parapatry. It is critical to understand how these traits interact upon secondary contact because some signals may promote reproductive isolation and speciation while others facilitate hybridization and introgression. Here, I investigated the relative functions of divergent song and plumage as potential isolating mechanisms between two subspecies of the red-backed fairy-wren (*Malurus melanocephalus*). In this species, song varies geographically and exhibits a pattern coincident with genetic differentiation across a historical biogeographic boundary. In contrast, plumage color exhibits a geographic pattern of variation that suggests asymmetrical introgression of red plumage from the *M. m. cruentatus* subspecies into the orange *M. m. melanocephalus* subspecies. Using song playbacks and artificially feathered mounts in a reciprocal experimental design including presentations of mismatched song and plumage, I tested the hypothesis that male aggressive response to these two divergent signals explains their observed patterns of introgression. Specifically, I predicated that males would discriminate on the basis of song, explaining why it has been resistant to introgression, whereas they would not discriminate on the basis of plumage, explaining why it has introgressed. In addition, I tested the hypothesis that asymmetrical introgression of plumage color is in facilitated by a behavioral asymmetry in territorial response between the different colored subspecies. I found that both subspecies discriminated strongly against foreign song irrespective of plumage color, but neither discriminated on the basis of plumage color when combined with local song. These results complement previous research indicating that song is often a key speciation phenotype between divergent taxa. I did not detect any asymmetrical territorial responses based on plumage color, so intersexual selection via female mate choice likely drives the previously documented introgression of red plumage color.

Introduction

When divergent taxa come into secondary contact, sexual signals may be important speciation phenotypes that can lead to assortative mating, reinforcement, and speciation (Ritchie 2007). However, if receiver response has not diverged in tandem with signals, response to these signals may lead to complex patterns of hybridization and introgression, particularly if there are asymmetrical preferences for traits associated with the heterospecific phenotype (Ryan and Wagner 1987; Veen et al. 2001; Stein and Uy 2006; Baldassarre and Webster 2013). In birds, song and plumage are known to be important species recognition traits that mediate reproductive isolation between many species pairs (Baker and Baker 1990; Baker and Boylan 1999; Patten et al. 2004; Edwards et al. 2005; Uy et al. 2009). However, several avian taxa exhibit asymmetrical responses to divergent song or plumage that may complicate or hinder assortative mating, potentially increasing gene flow and working against the speciation process (Stein and Uy 2006; Colbeck et al. 2010; Dingle et al. 2010; Baldassarre and Webster 2013). Importantly, the behavioral responses to these divergent signals must be studied in order to understand how they will affect the speciation process. In this way, investigators can move beyond documenting patterns of trait diversification and investigate the behavioral mechanisms that link trait divergence to speciation. Additionally, a phenotype is typically composed of several different traits, and understanding how these different signals interact when divergent populations come together is key because some may function as species barriers, while others introgress across genetic boundaries.

It is now widely recognized that most signaling systems involve multiple signals and often multiple modalities (Partan and Marler 1999; Rowe and Guilford 1999). The complexities of multimodal signaling have been well studied in many contexts, especially regarding the information contained in each signal, and whether multiple signals are redundant or independent (Uy and Safran 2013). However, less well understood is how multimodal signals interact in an evolutionary context, particularly how divergent signals of different modalities affect reproductive isolation and introgression. Of further interest is the possibility that divergent

signals may not only have different modalities but different modes of inheritance (e.g., cultural vs. genetic). How differential behavioral responses to these types of traits influence broad patterns of geographic variation and hybridization is poorly understood. Nonetheless, such scenarios are predicted to be common in birds, which exhibit social signals in visual and acoustic modalities, and often learn their songs (Catchpole and Slater 2008). Combining visual and acoustic modalities provides the potential for signals to evolve that target different receivers (e.g., males and females) and function at different spatial scales. Although divergence in multiple signal modalities has the potential for an additive effect on reproductive isolation (Baker and Baker 1990; Patten et al. 2004; Uy et al. 2009), such scenarios are particularly likely to lead to complex mating interactions between taxa and novel signal phenotypes when the individual signals do not all cause the same degree and direction of behavioral isolation (Greig and Webster 2013).

Here, I investigated the relative effectiveness and interactions of two divergent sexual signals to determine if broad geographic patterns of differential introgression across a hybrid zone could be explained by behavioral responses to the different signals. I studied this phenomenon in two subspecies of the red-backed fairy-wren (*Malurus melanocephalus*). In these two subspecies, song exhibits a geographic pattern of variation that is concordant with genetic divergence across a historical biogeographic barrier (Greig and Webster 2013), but plumage color variation suggests asymmetrical introgression of red plumage from the western *M. m. cruentatus* subspecies into the eastern, orange *M. m. melanocephalus* subspecies (Chapters 1 and 2; Baldassarre et al. 2013). This spatial decoupling is likely facilitated by the different inheritance patterns of song (learned) vs. plumage (genetic, Greig and Webster 2013) and a selective advantage through extra-pair mating to red males on the eastern side of the hybrid zone (Chapter 3; Baldassarre and Webster 2013), but the potential influence of behavioral responses on the relative geographic locations of song and plumage divergence are unknown. Using song playback, mount presentations, and a reciprocal experimental design, I assessed whether song or plumage was a more effective isolating mechanism, and determined if behavioral asymmetries in

aggressive interactions could explain the observed introgression of red plumage into orange populations. These experiments explicitly test the effect of divergent song and plumage on intrasexual (male-male) territorial interactions, allowing me to differentiate between the possibility that red males have a mating advantage because of female preference or increased competitive ability.

I tested two hypotheses regarding the effect of behavioral responses to these signals on patterns of trait introgression. The first hypothesis was that behavioral responses to the different traits would reflect the extent to which those traits had introgressed. Specifically, I predicted that behavioral response to song would hinder introgression of that trait because males would discriminate against foreign song. In contrast, I predicted that behavioral responses to plumage would not hinder introgression of that trait because males would not discriminate against foreign plumage. In both cases, I assume that a stronger response to the local signal is indicative of the potential for that signal to serve as a barrier to introgression and potentially gene exchange. This is because communication among males during territorial disputes is a key factor likely to determine whether a male can maintain a territory and subsequently mate successfully. For example, a lack of response by a territorial male to foreign song would suggest that resident males do not interpret males singing the foreign song type as conspecifics. Males signing the foreign song type would presumably be less likely to establish and defend a territory, attract females, and defend these females against intruders, resulting in low reproductive success. The second hypothesis was that behavioral responses to different traits would reflect the direction in which they have introgressed. Specifically, I predicted that behavioral responses to foreign song would be symmetrical between the two subspecies, reflecting the fact that song has not introgressed across the hybrid zone. In contrast, I predicted that behavioral responses to foreign plumage would be asymmetrical between the subspecies in a manner complementary to the eastward introgression of red plumage across the hybrid zone.

Methods

Study sites and general field methods

I conducted this research from October to December 2012 at two field site in Australia where color-banded populations of red-backed fairy-wrens have been monitored: the orange *melanocephalus* subspecies in Samsonvale, Queensland (hereafter Brisbane, GPS = 27°16'S, 152°51'E) and the red *cruentatus* subspecies in Coomalie Creek, Northern Territory (hereafter Darwin, GPS = 13°01'S, 131°12'E). These populations consisted predominantly of pairs without helpers and I specifically avoided testing any pairs with helpers to remove and effect of helper behavior on aggressive responses. In total I conducted 541 trials to 114 focal males, including those in both bright red/black and brown nuptial plumage (60 bright and 11 dull in Brisbane, 40 bright and 3 dull in Darwin). I noted the nesting stage of all focal pairs when possible (320/371 trials during known nesting stage in Brisbane, and all 170 trials during pre-nesting stage in Darwin as all males had established territories but not yet begun nesting during the study period). Although individual bird identity and nesting stage have potentially strong effects on territorial behavior, I tested focal males with as many experimental treatments as possible (up to six, described below) so that I could account for within-individual and between-nesting stage effects during statistical analyses.

Song playbacks and mounts

All vocalizations used for playback during experiments were recorded during natural dawn chorus displays using a Marantz PMD 661 solid-state digital recorder at 96 kHz sampling rate and 24-bit depth, or using a Marantz PMD 670 at 48 kHz sampling rate and 16-bit depth (D&M Professional, Itasca, IL) combined with ME66 shotgun microphones and K6 power modules with a frequency response of 0.04 – 20.0 kHz (Sennheiser Electronic Corporation, Old Lyme, CT). I used a total of 125 songs recorded from different individuals as playback stimuli: 44 from *M. m. cruentatus* populations, 46 from *M. m. melanocephalus* populations, and 35 from white-winged fairy-wrens (*M. m. leucopterus*, heterospecific control). Most recordings had been collected during two previous studies (Greig and Webster 2013; Greig et al. 2013), but I supplemented

these with more recordings collected during the experimental study period. I filtered out noise below 500 Hz and standardized the maximum amplitude of all playbacks using Raven 1.4 (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY). Playbacks consisted of the same song repeated at approximately 10 s intervals. I broadcasted playbacks using a portable amplified speaker with a frequency response of 0.1-12.0 kHz (Pignose Legendary 7-100, Pignose-Gorilla, Las Vegas, NV) and stored songs as uncompressed .WAV files on an iPod nano (Apple Inc., Cupertino, CA). I measured the amplitude of playbacks using a sound-pressure level meter (model number 33-2050, Radio Shack Corp., Fort Worth, TX), set at C-weighting, fast response (approximately 89.0 dB at one meter for all playbacks). This amplitude was similar to observed natural dawn song volume and was directly comparable to the protocol used in a previous study on this species (Greig and Webster 2013).

I created 12 artificially-feathered "quasi-taxidermic" mounts using lightweight Model Magic clay (Crayola LLC, Forks Township, PA) and colored feathers: four representing *M. m. cruentatus*, four representing *M. m. melanocephalus*, and four representing white-winged fairy-wrens (*M. leucopterus*, heterospecific control). For the red-orange back patch of the two red-backed fairy-wren subspecies, I affixed natural feathers collected from the Brisbane study population. These were left unaltered for the *melanocephalus* mounts, and colored with a non-toxic art marker for the *cruentatus* mounts according to the protocol described in Chapter 3 and Baldassarre and Webster (2013). I used this protocol because there is a demonstrable effect of this alteration on the spectral reflectance of feathers that accurately mimics the natural *cruentatus* plumage color and because I wanted this experiment to be directly comparable to the previous plumage manipulation experiment. I also affixed naturally collected tail feathers to the red-backed fairy-wren mounts. I used artificially colored synthetic black feathers for the remainder of the red-backed fairy-wren mounts, and blue and white artificial feathers for the white-winged fairy-wren mounts.

Experimental design

I conducted reciprocal playback experiments in the two study populations to examine the relative strengths and symmetry of territorial response to the two divergent signals. In each population I tested focal males with a series of six song and plumage combinations: 1) local song and local plumage, 2) local song and foreign plumage, 3) foreign song and local plumage, 4) foreign song and foreign plumage, 5) heterospecific song and local plumage, and 6) local song and heterospecific plumage. These trial combinations were designed to tease apart the independent effects and interactions of divergent song and plumage on behavioral response. I tested the same focal male with as many of the six treatments as possible to allow for within-individual analyses. Multiple trials to the same focal male were separated by at least one day, and the order of treatment types was balanced across the experiment. I tested a total of 61 males with all six treatments (44 in Brisbane, 17 in Darwin) and 53 males with 1-5 treatments (27 in Brisbane, 26 in Darwin).

After identifying a focal male and ensuring he was not engaged in a territorial interaction with a natural intruder, I would begin trial setup. During setup, I affixed the mount to a small tree or bush (referred to as "the mount bush") approximately 1-2 m off the ground within the focal male's territory in a manner mimicking the perch location of a typical intruder. The speaker was placed on the ground or embedded in the mount bush and lightly concealed with vegetation. The field observers would then retreat to a distance of approximately 15 m from the setup and conceal themselves behind vegetation. This setup typically lasted < 5 min, after which playback would begin immediately. Playback duration was dynamic and would continue until the focal bird arrived at the mount bush or < 1 m of the mount, after which time I would allow the playback to continue for three more songs. I would observe the focal male for 10 min after the playback was stopped, during which time the focal male was free to inspect the mount without any acoustic stimulus, and after which the trial was ended. If the focal male did not respond to the playback, I would discontinue the playback after 5 min. Thus, a trial could last as long as 15 minutes if the focal male responded at the very end of the maximum 5 min playback period. This protocol allowed me to measure male response to the acoustic stimulus of the playback before

encountering the mount, male response to the visual stimulus of the mount paired with the acoustic stimulus of the playback, and male response to the visual stimulus of the mount alone.

After all playbacks of foreign of heterospecific song in which the focal male did not respond, I played a local song to determine if the male was still within range of the speaker and that he was responsive to local playback. Males responded to these post-playback tests in 55% of trials (63/115 in Brisbane, 25/46 in Darwin), suggesting that in the majority of instances, the focal male could detect the foreign or heterospecific song but was simply not responding to it. Instances in which males did not respond to the post-playback test could either be cases where males moved out of range of the speaker during the 5 min playback of a foreign or heterospecific song (i.e., they may have been discriminating individuals that would have responded to the local song had they detected it), or were non-responsive to any playback type. Both of these scenarios likely contributed to the pool of individuals that did not respond to an initial local song, but I suspect the majority of these cases were due to truly non-responsive males because playback setup was very brief and individuals typically did not move out of the range of the speaker (as confirmed with visual sightings of the bird during and after the trial).

All trials were recorded with a Sony DCR-SX20 Handycam digital video camera (Sony Electronics, San Diego, CA) and later transcribed by two observers who were blind to the expectations of the various experimental treatments (see acknowledgements). During the trials, I also dictated behaviors and vocalizations into the camera to aid in transcription accuracy. The video observers recorded the presence of the focal male and latency to and duration of various behavioral responses (all variables listed in Table 4.1). The observers also recorded the same response variables for females that were present during the trial (females were present in 153/371 Brisbane trials and 41/170 Darwin trials). Because the observers recorded the time that each behavior occurred relative to the beginning of the trial (i.e., start of playback), I was able to distinguish between responses that occurred before the birds came within 10 m of the mount (and therefore more likely to be elicited by the acoustic stimulus) and those that occurred once the bird was within 10 m of the mount (and therefore more likely elicited by the visual stimulus). I

was also able to distinguish between responses that occurred before and after the playback had ended as described above.

Statistical analyses

All response variables violated the assumptions of normality, so I analyzed the data using generalized linear mixed models (GLMMs) using the package *lme4* in R v. 2.15 (R Core Team 2012). Preliminary model comparisons indicated that individual bird identity and playback order explained a significant amount of variation in response (results not shown), so I included them as random effects in all subsequent models. To collapse the many individual behavioral variables into a more manageable dataset, I used a principal components analysis (PCA) to generate two variables that described responses likely due to either the long-range acoustic playback stimulus (hereafter "approach/sing") or the close-range visual mount stimulus (hereafter "attack/inspect"). The approach/sing PCA consisted of six variables describing latency to approach to various distances and latency to vocal behavior, while the attack/inspect PCA consisted of three variables describing time spent within 5 m of the mount, latency to attack, and time spent attacking (Table 4.2). For both PCA analyses, PC1 explained the majority of the variation and was used as a response variable in subsequent GLMMs. Because approach/sing PC1 included several latency variables, and a low latency value indicates a strong behavioral response, I switched the sign for easier interpretation during analyses. I also added 1 to both approach/sing PC1 and attack/inspect PC1 to shift values out of the negative range because negative values are not permitted in the gamma distribution.

First, to compare the relative effects of the different song and mount types on behavioral response, I ran a global GLMM with approach/sing PC1 as a response variable, song and mount type as fixed effects, and a gamma distribution with an inverse link function. Second, to analyze only the effect of playback type on response strength, I restricted the dataset to trials with the local mount type and ran a GLMM with approach/sing PC1 as a response variable, playback type as a fixed effect, and a gamma distribution with an inverse link function. Finally, to analyze only

the effect of mount type on response strength, I restricted the dataset to trials where the male came to the mount bush and ran a GLMM with attack/inspect PC1 as a response variable, mount type as a fixed effect, and a Poisson distribution with a log link function. I ran each of these three GLMMs for both the Brisbane and Darwin populations separately.

Results

I found that in territorial interactions both subspecies discriminated against foreign songs irrespective of mount plumage. In the global model including both song and plumage type, males of both subspecies responded most strongly to local song and responded equally to the different mount types when song type was taken into account (Table 4.3, Figure 4.1). In the second model that included only trials with the local mount type, a similar pattern was observed. Focal males exhibited significantly stronger pre-arrival responses (i.e., approach/sing PC1) to local song, and there was no difference between strength of response to foreign and heterospecific songs (Table 4.3, Figure 4.2). Finally, for the subset of males that responded to song playback and approached the mount, responses predicted to be associated with visual stimulation (i.e., attack/inspect PC1) were not statistically different between the local and foreign mount types, but both were significantly higher than the response to the heterospecific mount in Darwin (Table 4.3, Figure 4.3). The results for this model in Brisbane were qualitatively similar, except that the difference between response strength to the local and heterospecific mounts was a non-significant trend in the same direction as in Darwin.

Discussion

Relative importance of song and plumage as isolating mechanisms

I found that red-backed fairy-wren subspecies on both sides of the hybrid zone discriminated strongly against foreign song but not plumage, supporting the hypothesis that behavioral responses to divergent signals reflect the degree to which they have introgressed. Specifically, in this system, behavioral response to song (i.e., discrimination against foreign song) should hinder

introgression of that trait, whereas behavioral response to plumage (i.e., lack of discrimination against foreign plumage) should allow introgression of that trait. This pattern suggests that male territorial response to foreign songs in the hybrid zone may serve as an isolating mechanism between the subspecies, but that male territorial response to foreign plumage is not a potent isolating mechanism. Regarding my second prediction, I did not find any behavioral asymmetries in plumage discrimination that would explain how male territorial interactions might facilitate the eastward introgression of red plumage into orange populations. This result suggests that factors other than male competition may be responsible for the asymmetrical introgression of red plumage. As suggested previously (Chapter 3; Baldassarre and Webster 2013), the fact that red males have a mating advantage in orange populations may be better explained by female preference for red plumage.

Function of short- vs. long-range multimodal signals

By analyzing pre- and post-arrival behavioral responses separately, I was able to discern that males do assess and respond to plumage color, but only after having responded to the song and approached the mount. Even then, males discriminated based on plumage color at the species level because they responded equally the local and foreign subspecies plumage, and only reduced their response when presented with the heterospecific mount type. At longer range, before approaching the mount, behavioral response was mediated completely by the acoustic signal of song. Males exhibited discrimination at the level of subspecies, responding most strongly to the local song, and responding to the foreign song as if it were a heterospecific song. Although I did not quantify this, it may be the case that song has diverged more extensively than plumage color in this system, making it a more effect signal for discriminating between subspecies. Additionally, these results support the idea that bird song is more often used as a signal mediating species interactions, whereas plumage color is typically used as a mate choice signal (Tobias et al. 2013). This phenomenon may be due to the modality of the two signals – males constantly advertise their presence on a territory even in the absence of a competitor in the

immediate area, while mate choice often takes place at close range at leks or display perches before copulation – but the generality of this idea is unclear.

The results of this study combined with the previous plumage manipulation experiment (Chapter 3; Baldassarre and Webster 2013) also suggest that the two different traits (song and plumage) present in this multimodal phenotype serve very different behavioral functions within a population. Song appears to function predominately as a signal of territory defense, a result supported by previous research on this species showing that duets and group songs are used most often in agonistic encounters with neighboring individuals (Dowling and Webster 2013). In contrast, this and several previous studies (Karubian 2002; Webster et al. 2008; Karubian et al. 2009; Baldassarre and Webster 2013) suggest that variation in plumage color is a key signal used by females when assessing potential mates. These within-population social behaviors likely shaped the evolution of this multimodal signal that later came to affect reproductive isolation and hybridization between the subspecies following secondary contact.

Future work

One novel aspect of this study is the breadth and level of detail of the behavioral responses that I quantified during the trials (Table 4.1). For the present analysis, I substantially reduced the complexity of the data using PCA, but these are several other avenues of research utilizing the more detailed behavioral data. For example, how males and females interact during territorial interactions is relatively unexplored in this species (but see Dowling and Webster 2013). In addition, this dataset offers the possibility to explore repeatability of male territorial response and examine potential implications for reproductive success. This will be the subject of a future study, as extensive population monitoring at the Brisbane study site allows for detailed quantification of several components of mating success that affect male fitness.

Conclusions

In summary, I found that behavioral responses of territorial males to divergent multimodal

signals can complement, if not functionally explain broad geographic patterns of differential introgression. My results are consistent with other work indicating that divergent song is often used as a species recognition signal and is thus resistant to introgression (Slabbekoorn and Smith 2002; Podos and Warren 2007), although this isolating mechanism has apparently not prevented introgression of plumage color in this system. In stark contrast, males of the two subspecies did not discriminate on the basis of plumage color, leaving it free to introgress, which is in contrast to previous work examining the influence of plumage on behavioral isolation in birds (Uy et al. 2009). Overall, therefore, this study suggests that differential responses to divergent signals of multiple modalities may cause complex behavioral interaction upon secondary contact that can lead to novel evolutionary trajectories. Most importantly, I have shown that divergent social signals do not always lead to reproductive isolation and speciation; it is critical to consider both the behavioral responses to signals and the possibility that multimodal signals may elicit different responses within a single organism's phenotype.

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Table 4.1 All the behavioral responses quantified in this study. These behaviors can be broadly assigned to four different categories that describe a male's territorial response.

Behavior	Category
Latency to 10 m	Approach distance
Time < 10 m	Approach distance
Latency to 5 m	Approach distance
Time < 5 m	Approach distance
Latency to mount bush	Approach distance
Time in mount bush	Approach distance
Latency to 0.5 m	Approach distance
Time < 0.5 m	Approach distance
Latency to song	Vocal behavior
Number of songs	Vocal behavior
Duration of chatter call	Vocal behavior
Duration of whining call	Vocal behavior
Duration of contact call	Vocal behavior
Duration of buzz call	Vocal behavior
Latency to attack	Attacking behavior
Time attacking	Attacking behavior
Number of fly-overs	Attacking behavior
Time < 6 in of mate	Interaction with mate
Time allopreening	Interaction with mate
Number of hops over mate	Interaction with mate

Table 4.2 A summary of principal components analyses (PCAs). The first PCA includes behaviors likely to be stimulated by the acoustic signal before arrival, whereas the second PCA includes behaviors only exhibited after arrival and during visual inspection of the mount.

PCA: approach/sing	PC1	PC2	PC3
Eigenvalue	3.0389	0.9702	0.7902
Cumulative percentage	50.648	66.817	79.986
Factors		Loadings	
Latency to 10 m	0.47183	-0.1232	-0.01662
Latency to 5 m	-0.25342	0.77789	0.25392
Latency to mount bush	0.48849	0.38687	-0.09621
Latency to 0.5 m	0.4614	0.41171	-0.22798
Latency to male song	0.40356	-0.21873	-0.16119
Latency to duet	0.31429	-0.11266	0.92089

PCA: attack/inspect	PC1	PC2	PC3
Eigenvalue	2.2844	0.4469	0.2686
Cumulative percentage	76.148	91.046	100
Factors		Loadings	
Time within 0.5 m	0.56518	-0.70041	0.43589
Time attacking	0.56371	0.71366	0.41584
Latency to attack	-0.60234	0.01069	0.79817

Table 4.3 Results of generalized linear mixed models of the effect of song and mount type on behavioral response. Three separate GLMMs were run: a global model with both song and mount type, a reduced model with only song type given local mount, and a reduced model with only mount type given prior response to the playback and approach to the mount. Significant effects are highlighted in bold.

Model	Fixed effect	Estimate		*t*		*p*	
		Darwin	Brisbane	Darwin	Brisbane	Darwin	Brisbane
Global: song and plumage	Intercept	0.9	0.84	2.99	4.65	**< 0.001**	**< 0.001**
	L song	-0.76	-0.47	-2.82	-3.1	**< 0.01**	**< 0.01**
	F song	0.67	-0.18	1.07	-1.1	0.28	0.28
	L mount	0.12	-0.002	0.63	-0.03	0.53	0.98
	F mount	0.35	-0.03	1.32	-0.29	0.19	0.77
Response to song given local mount	Intercept	0.17	0.2	21.74	19.91	**< 0.001**	**< 0.001**
	L song	0.03	0.04	2.84	2.98	**< 0.01**	**< 0.01**
	F song	0.01	0.01	0.66	0.61	0.51	0.54
Response to mount given approach	Intercept	-0.82	-0.17	2.52	-0.81	**0.01**	0.42
	L mount	0.65	0.46	1.29	2.18	0.2	**0.03**
	F mount	1.19	0.43	2.52	2.09	**0.01**	**0.04**

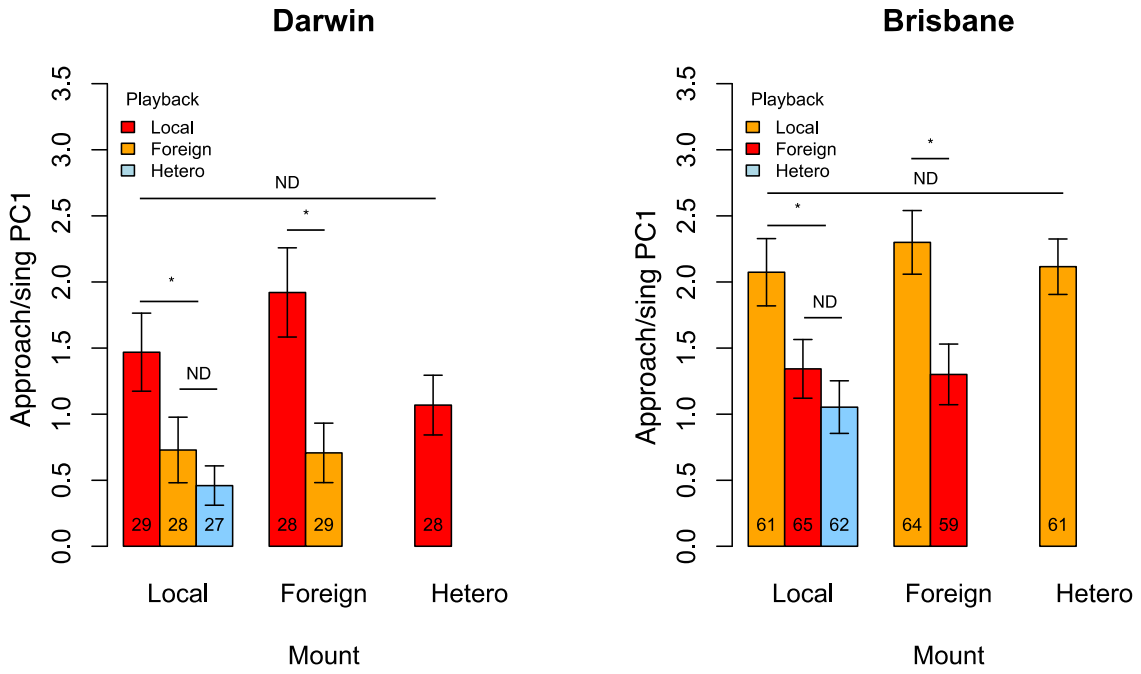

Figure 4.1 Results of the global generalized linear mixed model with both song playback and mount type. The left panel shows the results for Darwin where the local plumage is red, and the right panel shows the results for Brisbane where the local plumage is orange.

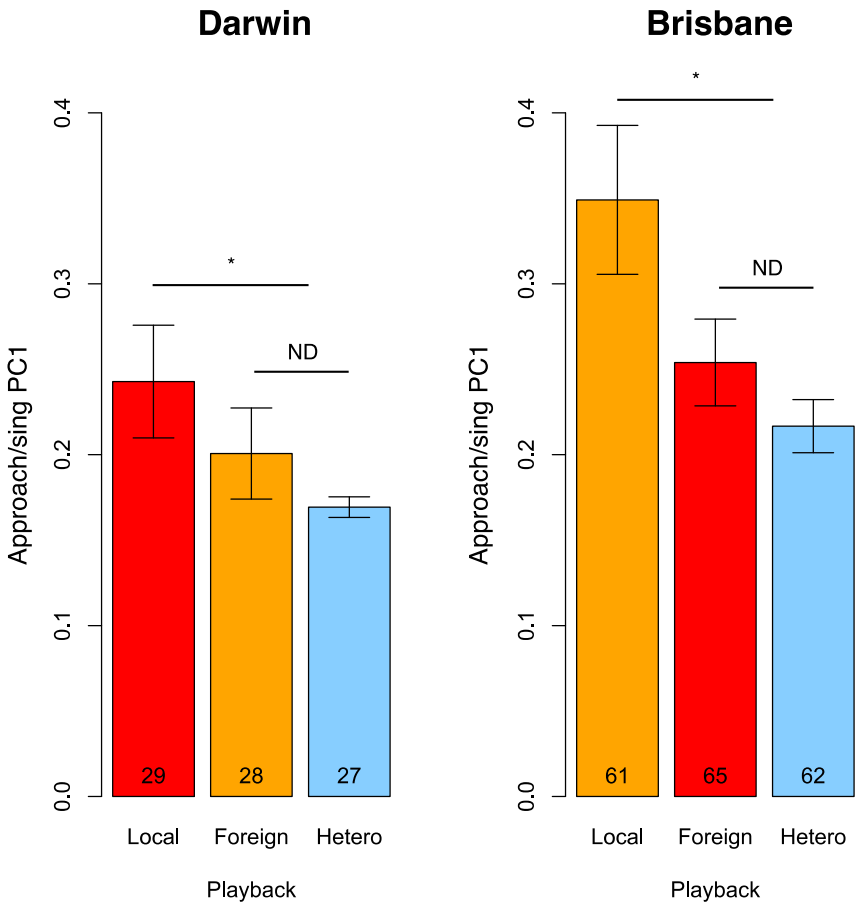

Figure 4.2 Results of a generalized linear model of the effect of playback type on pre-arrival response given local mount type. The left panel shows responses from Darwin where the local song is represented in red, and the right panel shows responses from Darwin where the local song is represented in orange.

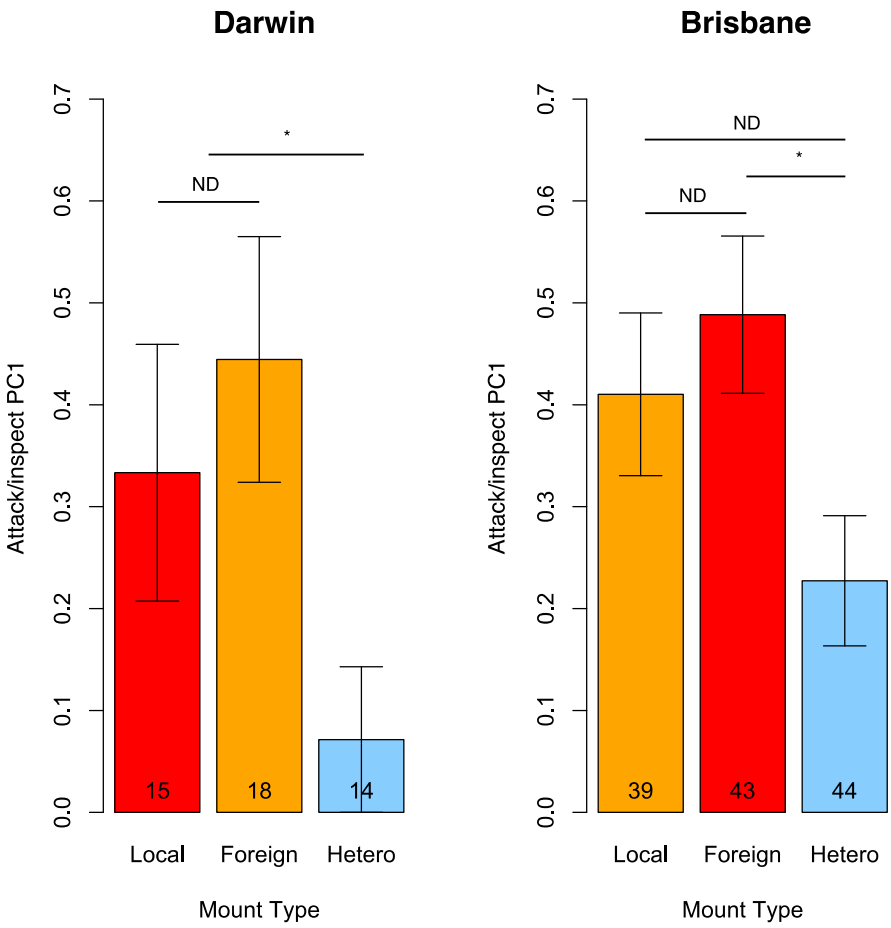

Figure 4.3 Results of a generalized linear mixed model of the effect of mount type on close-range behavioral response given prior response to playback and approach to the mount bush. The left panel shows responses in Darwin where the local plumage color is red, and the right panel shows responses in Brisbane where the local plumage color is orange.