



Differential rates of phenotypic introgression are associated with male behavioral responses to multiple signals

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Received January 28, 2015

Accepted August 12, 2015

Sexual selection on multiple signals may lead to differential rates of signal introgression across hybrid zones if some signals contribute to reproductive isolation but others facilitate gene flow. Competition among males is one powerful form of sexual selection, but male behavioral responses to multiple traits have not been considered in a system where traits have introgressed differentially. Using playbacks, mounts, and a reciprocal experimental design, we tested the hypothesis that male responses to song and plumage in two subspecies of red-backed fairy-wren (*Malurus melanocephalus*) explain patterns of differential signal introgression (song has not introgressed, whereas plumage color has introgressed asymmetrically). We found that males of both subspecies discriminated symmetrically between subspecies' songs at a long range, but at a close range, we found that aggression was equal for both subspecies' plumage and songs. Taken together, our results suggest that male behavioral responses hinder the introgression of song, but allow for the observed asymmetrical introgression of plumage. Our results highlight how behavioral responses are a key component of signal evolution when recently divergent taxa come together, and how differential responses to multiple signals may lead to differential signal introgression and novel trait combinations.

KEY WORDS: Asymmetrical behavior, differential introgression, male competition, multimodal signals, sexual selection, song.

Sexual signals mediate competitive and reproductive interactions between individuals, and accordingly have the potential to affect reproductive isolation between populations (Coyne and Orr 2004; Price 2008b). However, the effects of a sexual signal on reproductive isolation will depend on the behavioral responses of conspecifics to it, because those responses will determine whether the signal limits or increases gene flow across a contact zone (Kaneshiro 1980; Ryan and Wagner 1987; Veen et al. 2001; Stein and Uy 2006; Ritchie 2007; Price 2008b). Typically, an individual is predicted to respond most strongly, in both competitive and mating interactions, to traits of its own population over traits from another population, slowing or preventing gene flow across a contact zone (Baker and Baker 1990; Patten et al. 2004; Uy et al. 2009; Vortman et al. 2013). However, in some

cases, individuals respond equally or more strongly to signals from distant/divergent populations, potentially increasing gene flow and leading to asymmetrical introgression of the divergent signal and genes linked to it (Ryan and Wagner 1987; Grant and Grant 1997; Bronson et al. 2003; Stein and Uy 2006; Baldassarre and Webster 2013). Differential behavioral responses to multiple signals may therefore lead to novel combinations of traits and complex evolutionary consequences, including the formation of hybrid species, if some signals introgress while others do not (Grether et al. 2009; Bro-Jorgensen 2010; Brelsford et al. 2011; Abbott et al. 2013; Amorim et al. 2013; Greig and Webster 2013).

Competition among males is one powerful form of selection that may impact patterns of hybridization and introgression (reviewed in Qvarnström et al. 2012). In birds, divergent (e.g., foreign or heterospecific) acoustic signals typically hinder territorial interactions among males, and therefore may slow gene flow

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between populations (Baker and Mewaldt 1978; Baker 1991; Searcy et al. 2002; Slabbekoorn and Smith 2002; Patten et al. 2004; Edwards et al. 2005; Podos et al. 2007). Indeed, when males from divergent populations compete for territories or mates, acoustic signals often converge to promote communication (Baker and Boylan 1999; Qvarnström et al. 2006; Price 2008a; Grether et al. 2009; Tobias et al. 2013). However, divergent visual signals sometimes facilitate gene flow or introgression. For example, in *Ficedula* flycatchers, divergent (brown) plumage allows males to establish territories among heterospecifics because brown males receive less aggression, but they can still attract mates and defend territories by singing the conspecific song (Qvarnström et al. 2006; Vallin et al. 2012). In *Manacus* manakins, divergent heterospecific plumage traits are preferred by females, leading to asymmetrical introgression of plumage from one population to another (McDonald et al. 2001; Stein and Uy 2006). In *Setophaga* warblers, males that have divergent introgressing plumage traits are more aggressive overall and more successful at territory establishment (Pearson and Rohwer 2000). Taken together, these studies suggest that divergence in acoustic signals tends to hinder communication and gene flow, but divergence in visual signals may in some circumstances enhance gene flow, particularly when paired with a nondivergent song.

Systems in which there is asymmetrical introgression of sexual signals from one species/subspecies into the genetic background of another (e.g., Parsons et al. 1993; Rohwer et al. 2001; Baldassarre et al. 2014) provide an opportunity to examine how the introgression of a divergent trait is influenced by male behavioral responses to it. However, the role of differential male behavioral responses to multiple sexual signals has not been examined in a system where some signals have introgressed, but others have not. Here, we test male responses to divergent visual and acoustic signals in a system where plumage has introgressed, but song has not. In red-backed fairy-wrens (*Malurus melanocephalus*), there are two subspecies that show genetic differentiation across the Carpentarian Barrier, a historical geographic boundary (Lee and Edwards 2008; Fig. 1). This boundary is porous, however, with high levels of gene flow, suggesting that periods of allopatry were followed by secondary contact and hybridization (Baldassarre et al. 2014). Song exhibits a geographic pattern of variation coincident with the historical contact zone and location of greatest genetic differentiation (Greig and Webster 2013; Fig. 1). Plumage, on the other hand, shows a geographic pattern of variation suggestive of asymmetrical introgression from one subspecies (*M. m. cruentatus*: red) to the other (*M. m. melanocephalus*: orange) (Baldassarre et al. 2013, 2014; Fig. 1). Experimental plumage manipulations in *M. m. melanocephalus* populations have shown that visually *cruentatus*-like males (dyed red) have greater extra-pair mating success than do *melanocephalus*-like males (orange) (Baldassarre and Webster 2013), indicating that

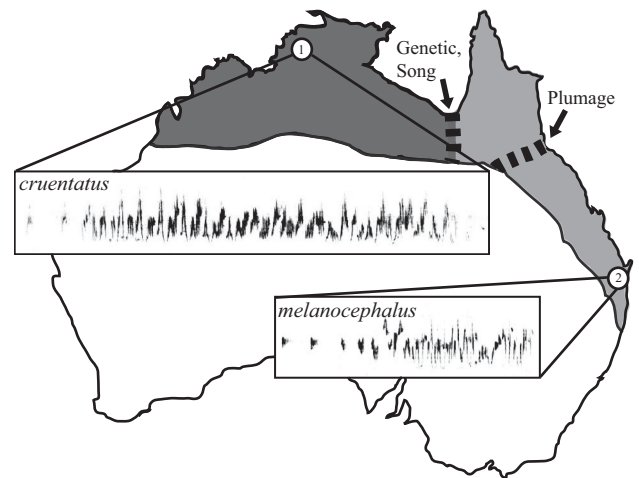


Figure 1. Study site locations (1 = Coomalie Creek, 2 = Samsonvale) relative to the approximate locations of genetic, song and plumage divergence. Example spectrograms are shown from both sites, and time scales for both are equivalent (song length = ~6 s for *cruentatus*, ~3.5 s for *melanocephalus*). Dark gray region to the west of the “Genetic, Song” dotted line (near the Carpentarian Barrier) indicates approximate range of genetic *cruentatus* subspecies, all with red plumage and *cruentatus*-like songs. Pale gray region between the dotted lines indicates genetic *melanocephalus* subspecies with red plumage and *melanocephalus*-like songs. Pale gray region to the south of the plumage dotted line indicates genetic *melanocephalus* subspecies with orange plumage and *melanocephalus*-like songs.

red males have a reproductive advantage in orange populations. However, the mechanism of this advantage is not clear; it may be due to female preferences for red or to differential male responses to red during competitive interactions. Additionally, playback experiments have shown that males discriminate against songs from the opposite subspecies in territorial interactions (Greig and Webster 2013), but it is not known how plumage impacts acoustic discrimination; it may either facilitate or hinder differential acoustic response. Here, we assessed male territorial response to divergent plumage and song simultaneously, taking into consideration the fact that song is a longer ranging signal than plumage and is therefore assessed first in territorial interactions (Uy and Safran 2013).

Using feathered decoys (mounts), song playbacks, and a reciprocal experimental design with different combinations of song and plumage, we tested the hypothesis that male behavioral discrimination differs between song and plumage, complementing and potentially facilitating the observed pattern of differential introgression. If the different introgression patterns of song and plumage can be explained by male–male interactions, then we predicted that male responses to divergent song would be symmetrical between subspecies, and that male responses to divergent plumage would be asymmetrical between subspecies. Alternatively, if male–male interactions have actively hindered or

have not influenced introgression of plumage, then we predicted that male responses to divergent plumage would be symmetrical or neutral between subspecies.

Specifically, we first asked if subspecies discriminated symmetrically against divergent songs at a long range, potentially hindering song introgression. We predicted that if homotypic (i.e., same subspecies, hereafter “own”) song is advantageous for signalers, then males of both subspecies should be more likely to respond to their own subspecies song than to songs from the other subspecies (hereafter, “other”), because their own subspecies songs should be a greater territorial threat. Second, in situations where males responded to song and approached mounts to a close range, we asked if male aggression could affect introgression of traits. We predicted that if singing their own subspecies song is advantageous for signalers, then males of both subspecies should respond to divergent songs in a symmetrical manner; we predicted that males would be more aggressive towards mounts paired with their own subspecies songs than to mounts paired with the other subspecies songs, again because their own subspecies’ songs should be a greater threat in territorial intrusions. We predicted that if red plumage is advantageous for signalers in territorial interactions, then males of both subspecies should respond to divergent plumage in an asymmetrical manner; specifically that males of both subspecies would be more aggressive toward mounts with red plumage, because red plumage should be a greater territorial threat. Although we predicted that stronger responses would be associated with advantageous signals, an alternative possibility is that weaker responses are associated with advantageous signals if receiving less aggression allows males to establish territories more readily (e.g., Vallin et al. 2012). In either case, it is the symmetry or asymmetry of male responses, irrespective of the directionality of those responses that allow us to support or refute our primary hypothesis that male behavioral responses complement differential patterns of signal introgression.

Material and Methods

STUDY SPECIES

Red-backed fairy-wrens are insectivorous passerines that inhabit open forests in northern and eastern Australia (Rowley and Russell 1997). They are resident cooperative breeders (Rowley and Russell 1997) with female-biased dispersal; males often disperse only as far as a neighboring territory (Varian-Ramos and Webster 2012). Territories are typically small (e.g., 1 ha; Rowley and Russell 1997) and extra-pair paternity is common, occurring in up to 63% of broods (Webster et al. 2008). Males exhibit delayed plumage maturation, retaining female-like brown plumage either as auxiliary helpers or as primary breeders for multiple seasons before adopting red-black nuptial plumage (Rowley and Russell 1997; Webster et al. 2010). Both males and females sing

a typical *Malurus* song, notably during dawn chorus displays and territorial interactions (Dowling and Webster 2013; Rowley and Russell 1997). These songs differ between the two subspecies in characteristics such as length, note rate, and frequency (Greig and Webster 2013). Both sexes also give a suite of calls in social contexts, but nothing is known about how these calls vary between the two subspecies (Rowley and Russell 1997). Songs are likely learned from socially associated individuals, as in the splendid fairy-wren (*M. splendens*) (Greig et al. 2012), although song inheritance has not been explicitly studied in red-backed fairy-wrens. Plumage, in contrast, is likely inherited genetically, with minimal environmental contributions (Baldassarre et al. 2013).

STUDY SITES AND GENERAL FIELD METHODS

We worked from October to December of 2012 at two sites in Australia with color-ringed study populations: (1) Coomalie Creek, Northern Territory; GPS = 13°01’S, 131°12’E (*M. m. cruentatus*) and (2) Samsonvale, Queensland; GPS = 27°16’S, 152°51’E (*M. m. melanocephalus*) (Fig. 1). Baldassarre and Webster (2013) describe details of population monitoring and banding procedures. These populations consisted primarily of pairs without helpers, and we specifically avoided testing pairs with helpers to remove any effect helpers may have had on territorial response. We tested pairs that had either red-black or brown breeding males (five brown and 70 red-black *melanocephalus* focal males, three brown and 40 red-black *cruentatus* focal males) and we noted the nesting stage of all focal pairs when possible (68/75 *melanocephalus* focal males with known nesting stages, and 43/43 *cruentatus* focal males in the prenesting stage). Sample sizes differ between the two sites due to the amount of time spent at each site and the relative ease of locating birds at the respective sites (*cruentatus* males were generally less vocal and more difficult to locate). Although bird identity and nesting stage have potentially strong effects on response strength, we tested focal males with as many experimental treatments as possible (up to the maximum of all six treatment types, described below) to include within-individual and between-nesting stage effects in our analyses. Females were present in 41 of 169 trials to *cruentatus* and 153 of 372 trials to *melanocephalus*; male response was highly correlated with female response in both populations (analyses not shown), which is consistent with previous work showing that pairs collaborate in territory defense (Dowling and Webster 2013; Greig and Webster 2013). Females almost never responded alone (14/236 trials in which males did not respond), but males would often respond alone (125/347 trials in which females did not respond), which suggests that male response was not entirely dependent upon female response. Nonetheless, we included female presence in our analyses (described below) to account for the potential influence of females on male response.

PLAYBACKS AND MOUNTS

All vocalizations used for playbacks were recorded during natural dawn chorus displays using a Marantz PMD 661 solid-state digital recorder at 96 kHz sampling rate, 24-bit depth, or using a Marantz PMD 670 at 48 kHz sampling rate, 16 bit-depth (D&M Professional, Itasca, Illinois), combined with ME66 shotgun microphone capsules and K6 power modules (Sennheiser Electronic Corporation, Old Lyme, Connecticut; frequency response 0.04–20.0 kHz). We used a total of 126 songs recorded from different individuals for playback: 44 from *M. m. cruentatus* populations (13 from Coomalie Creek, 31 from three other *cruentatus* populations), 47 from *M. m. melanocephalus* populations (nine from Samsonvale, 38 from three other *melanocephalus* populations) and 35 from white-winged fairy-wrens (*M. leucopterus*; an allopatric heterospecific sister species control). We controlled for playback ID and playback origin population by including both as random effects in our analyses (detailed below; see Table S1 for details on playback origin). Additionally, when playing songs from within the same population to focal birds, we chose songs recorded from different areas of the study site to minimize the likelihood that birds were familiar with the playback stimulus. Playbacks consisted of the same song repeated at intervals of approximately 10 s. We filtered out noise from all playbacks below 500 Hz and we standardized the maximum amplitude of playbacks in Raven 1.4 (Bioacoustics Research Program 2004) so that all playbacks were of comparable volume. We used an amplified speaker with a frequency response that encompassed typical red-backed fairy-wren songs (Greig et al. 2013) (Pignose Legendary 7–100, Pignose-Gorilla, Las Vegas, NV; frequency response, 0.1–12.0 kHz) and an iPod nano (Apple Inc., Cupertino, CA) with uncompressed .WAV files for playback. We tested the amplitude of playbacks with the field playback equipment using a sound-pressure level meter (model number 33–2050, Radio Shack Corporation, Fort Worth, Texas), 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 levels and was comparable to the protocol used in a previous study (Greig and Webster 2013).

We created 12 artificial mounts using lightweight clay and colored feathers: four representing *M. m. cruentatus*, four representing *M. m. melanocephalus*, and four representing white-winged fairy-wrens (*M. leucopterus*; our heterospecific control), all in nuptial male plumage. For the red or orange back patch of the red-backed fairy-wren mounts, we used natural feathers collected from the Samsonvale study population; these were left unaltered for the orange *M. m. melanocephalus* mounts, and were colored red with a marker for the red *M. m. cruentatus* mounts according to the protocol described in Baldassarre and Webster (2013). We used this protocol because there is a demonstrable effect of this color manipulation on the spectrographic profile of feathers that mimics natural *M. m. cruentatus* plumage, and

because we wanted this experiment to be directly comparable to the previous plumage manipulation experiment in which red-painted males had higher extra-pair mating success. We did not color orange feathers with a sham marker because such manipulation did not have any effect on the spectrographic profile of feathers, nor mating success, in the previous experiment (Baldassarre and Webster 2013). We used artificially colored black feathers for the remainder of the red-backed fairy-wren mounts. We used artificially colored blue and white feathers in an analogous bicolored pattern for the white-winged fairy-wren mounts.

EXPERIMENTAL DESIGN

We conducted reciprocal playback experiments in the two study populations to quantify the strength and symmetry of male response. In each population we tested focal males with a series of six song and plumage combinations: (1) own subspecies song and plumage, (2) own subspecies song and other subspecies plumage, (3) other subspecies song and own subspecies plumage, (4) other subspecies song and plumage, (5) heterospecific song and own subspecies plumage, and (6) own subspecies song and heterospecific plumage. Note that in this study, we use the term “own” to refer to any song from within the same genetic subspecies, but not necessarily the same population, as the focal bird. Similarly, we use the term “other” to refer to any song from the other genetic subspecies. These trial combinations allowed us to tease apart the independent effects of divergent song and plumage, and also to compare these effects between the two subspecies (as in Baker and Baker 1990; Uy et al. 2009; McEntee 2014). We tested each male with as many of the six treatment types as possible so that we could quantify within-individual variation in response. Multiple presentations to the same individual were separated by at least one day and the order of treatment types was balanced across all individuals in the experiment. We tested a total of 63 males with all six trial combination types (17 *cruentatus* and 46 *melanocephalus*) and 55 males with 1–5 combination types (26 *cruentatus* and 29 *melanocephalus*), for a total of 541 trials. The number of trials varied between individuals simply because some individuals were more difficult to locate than others or were discovered later in the season, preventing us from having the opportunity to conduct all six trials. We accounted for this unbalanced sampling using a GLMM approach (detailed below).

Trial set-up began after a focal male had been identified and if the male was not engaged in other territorial interactions with natural intruders. During set-up, a mount was wired 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 a perch location of a natural intruder. The speaker was placed on the ground below the mount and lightly concealed with vegetation. Observers moved to a distance of approximately 15 m from the set-up and concealed themselves behind vegetation. Set-up duration

Table 1. Behavioral responses quantified in this study and their loadings on the first PCA score, which we used as a measure of response intensity.

	PC1
Eigenvalue	2.47
Percent variation	43.6
Time spent 10 m	0.33
Time spent 5 m	0.36
Time spent mount bush	0.37
Time spent 0.5 m	0.35
Time spent attacking	0.21
Latency 10 m	-0.17
Latency 5 m	-0.27
Latency mount bush	-0.34
Latency 0.5 m	-0.34
Latency to attack	-0.27
No. male songs	0.10
No. duets	0.11
Latency male song	-0.14
Latency duet	-0.10

was consistently <5 minutes, after which the observers would immediately begin the playback. Playback duration was variable up to a maximum of 5 minutes. We continued playback until the focal bird came to the mount bush or <1 m from the mount; from that time, we would allow the playback to continue for three more songs. This protocol allowed us to assess male responses to the mount after a constant number of songs had been played while the male was in close proximity. We observed the focal male for 10 minutes after the playback had stopped. If the focal male did not respond to the playback, we discontinued the trial after 5 minutes.

We recorded all trials with a video camera (Sony DCR-SX20 Handycam digital video camera; Sony Electronics, San Diego, CA) and we dictated behaviors and vocalizations into the camera during the trials. Two observers who were blind to the expectations of the treatment types transcribed the males' responses. We recorded the latency to, and duration of, focal male responses associated with approaching the mount, attacking the mount, and singing (all responses listed in Table 1).

STATISTICAL ANALYSES

We considered two tiers of response in our analyses. First, to assess response to song as a long range signal irrespective of mount color, we classified all trials (372 to *melanocephalus* and 169 to *cruentatus*) as either having any approach response behavior or not (0 or 1); any male approach to within 10 m was considered a positive ("1") response. Because birds classified as having a "0" response did not approach the mount within a distance of 10 m,

this binomial response was likely associated with long-distance recognition of song rather than visual recognition of plumage.

Second, to quantify aggression given that a male had an opportunity to visually assess a mount, we used the subset of trials in which birds had approached to a distance within 10 m of the mount (198 to *melanocephalus* and 63 to *cruentatus*). We classified these trials as either having any inspection/attack behavior or not (0 or 1); any approach to within 0.5 m of the mount or physical attack of the mount was considered a positive ("1") response. Additionally, we used a principal components analysis (PCA) on the correlation matrix of all response variables to generate a score of overall response strength for this subset of trials.

We analyzed responses using generalized linear-mixed models (GLMMs) implemented in package *lme4* in R v. 2.15 (R Core Development Team 2008). To account for within-individual effects and playback/mount exemplar effects, we included male ID, playback ID, mount ID, and playback origin population as random effects in all models. For binary responses we used mixed logistic regression models, using binomial distributions and logit link functions. For PC1 scores we used linear-mixed models. Preliminary model comparisons indicated that playback order and nesting stage did not explain significant variation in any models, so we excluded these effects in our final analyses. We included female presence in all models of male response given that males approached to within 10 m.

Results

PC1 explained 43.6% of the variation in our response variables (Table 1). Variables associated with time spent at different distances from the mount were necessarily correlated because time spent within a small radius of the mount was included in the time spent within a larger radius. Similarly, latency to approach within a small radius necessarily encompassed the latency to approach within a larger radius. Overall, higher values of PC1 indicated birds that approached more quickly, stayed closer to the mount for longer durations, and sang more songs more promptly.

Males were more likely to respond to (approach) own subspecies songs than other subspecies songs irrespective of mount plumage type in both populations (Table 2, Fig. 2A, B). Males of both subspecies were equally unlikely to respond to other subspecies songs and heterospecific controls, suggesting that they did not consider other subspecies songs any more of a threat than an allopatric heterospecific song (Table 2, Fig. 2A, B). However, responses to other subspecies songs and heterospecific songs were nonzero, and were relatively high in *melanocephalus*, with approximately 40% of males responding to presumably unfamiliar *cruentatus* and white-winged fairy-wren songs.

For the subset of males that approached mounts to within 10 m, male inspection and attack behavior was equal for red and

Table 2. Results of GLMMs relating male territorial response strength of each focal subspecies to different song and plumage treatments (i.e., playbacks and mounts).

Response	Fixed effects ¹	focal: <i>cruentatus</i>			focal: <i>melanocephalus</i>		
		Estimate	<i>z/t</i>	<i>P</i>	Estimate	<i>z/t</i>	<i>P</i>
Proportion approaching	Intercept	-1.03	-2.55	0.011	-0.36	-1.13	0.260
<i>Song</i>	Own vs Other	1.61	3.61	< 0.001	1.22	3.90	< 0.001
	Own vs Hetero	2.44	3.23	0.001	1.46	3.41	< 0.001
	Other vs Hetero	-0.84	-1.08	0.281	-0.25	-0.58	0.562
<i>Plumage</i>	Own vs Other	-0.39	-0.89	0.371	-0.13	-0.41	0.683
	Own vs Hetero	0.17	0.33	0.743	-0.42	-0.99	0.321
	Other vs Hetero	-0.56	-1.06	0.290	0.29	0.68	0.500
Proportion inspecting/attacking (given approach)	Intercept	-1.16	-1.08	0.279	-1.28	-2.31	0.021
	Female presence	0.77	0.84	0.402	1.51	3.22	0.001
<i>Song</i>	Own vs Other	-0.31	-0.31	0.756	-0.38	-0.86	0.392
	Own vs Hetero	0.39	0.22	0.830	0.11	0.16	0.870
	Other vs Hetero	0.06	0.03	0.974	-0.49	-0.71	0.480
<i>Plumage</i>	Own vs Other	-0.48	0.56	0.577	0.13	0.31	0.758
	Own vs Hetero	-2.49	1.67	0.136	1.47	2.54	0.011
	Other vs Hetero	-2.96	-1.80	0.072	-1.34	-2.34	0.019
Response intensity (given approach)	Intercept	-1.04	-1.51	0.131	-1.20	-2.90	0.003
	Female presence	0.19	0.35	0.723	1.41	4.66	< 0.001
<i>Song</i>	Own vs Other	0.53	0.68	0.494	0.27	0.84	0.401
	Own vs Hetero	1.21	0.89	0.374	0.64	1.40	0.140
	Other vs Hetero	-0.68	-0.49	0.626	-0.38	-0.78	0.435
<i>Plumage</i>	Own vs Other	-0.15	-0.28	0.783	0.14	0.47	0.637
	Own vs Hetero	1.27	1.89	0.059	0.54	1.47	0.142
	Other vs Hetero	-1.42	-2.20	0.028	-0.40	-1.08	0.282

¹Bird ID, playback ID, mount ID, and playback origin incorporated into all models as random effects. "Own" refers to song or plumage from the focal birds' own subspecies, "Other" refers to song or plumage from the other subspecies, and "Hetero" refers to white-winged fairy-wren song or plumage. *P*-values are for comparisons of the fixed effects, and values <0.06 are highlighted in bold to illustrate significant and marginally nonsignificant results.

orange mounts and was equal across song types in both populations, although *melanocephalus* males were less likely to inspect/attack heterospecific control mounts (Table 2, Fig. 2C, D). Similarly, response intensity (PC1) given approach was equal for red and orange mounts and was equal across song types in both populations, although *cruentatus* males were less aggressive to heterospecific control mounts (Table 2, Fig. 3). Overall, therefore, once a male had an opportunity to see a red or orange mount (by approaching to within 10 m), the level of aggression to that intruder was equal across song and mount types in both populations.

Female presence had a significant effect on male likelihood of inspection/attack behavior and on the strength of male response in *melanocephalus* but not *cruentatus*; *melanocephalus* males were more likely to attack, and their response strength was stronger, when females were present (Table 2).

In summary, males of both subspecies discriminated between their own subspecies and the other subspecies songs when deciding whether or not to initially approach. However, given that

a male approached and had an opportunity to visually assess a red or orange mount, the likelihood of inspecting/attacking that mount was independent of both song playback and mount plumage type.

Discussion

BEHAVIORAL RESPONSES TO SONG AND PLUMAGE

We found that red-backed fairy-wren subspecies on both sides of the hybrid zone discriminated between their own subspecies and the other subspecies songs, but not between their own subspecies and the other subspecies (red vs orange) plumage, supporting the hypothesis that behavioral responses to divergent signals impact the degree to which those signals can introgress. In this case, male symmetrical discrimination against the other subspecies song has the potential to actively restrict song introgression, whereas the lack of discrimination between red and orange plumage should permit (but not enhance) asymmetrical introgression of plumage traits. However, our results were different from our predictions in

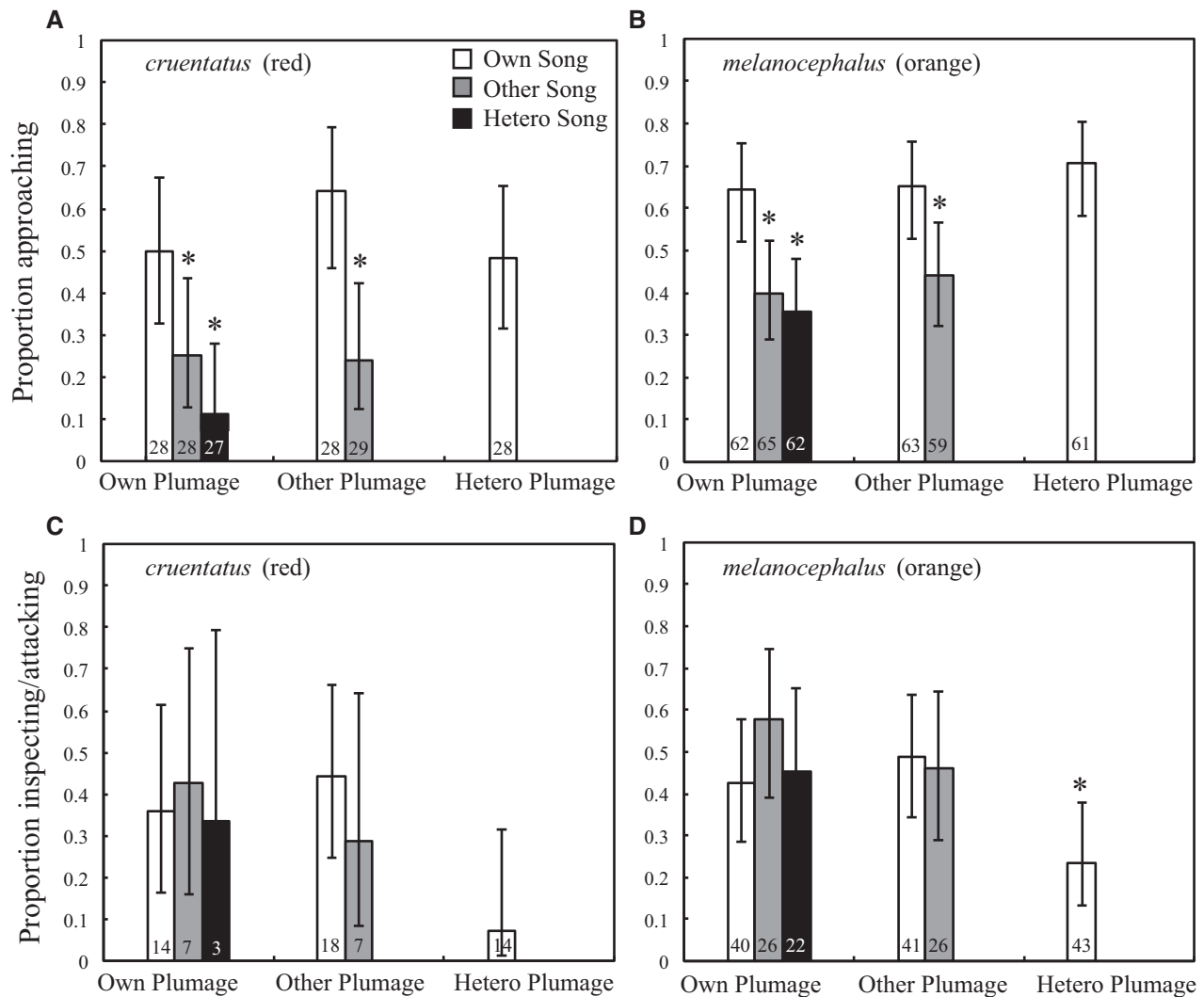


Figure 2. Binomial responses to the different mount and playback types for males of each focal subspecies. Panels A and B indicate proportion of males of each focal subspecies responding to the mount/playback by approaching to within 10 m. Panels C and D indicate the proportion of males of each focal subspecies inspecting or physically attacking the mount, given that males approached to within 10 m (the subset of males that responded in A and B). "Own" refers to song or plumage from the focal birds' own subspecies, "Other" refers to song or plumage from the other subspecies, and "Hetero" refers to white-winged fairy-wren song or plumage. Sample sizes are given within bars. Error bars are binomial 95% confidence intervals. Asterisks indicate treatments that were significantly different (detailed in Table 2).

several respects and highlight unexpected and subtle mechanisms by which behavioral responses may influence signal introgression.

Males of both subspecies were more likely to respond to their own subspecies songs than to the other subspecies or heterospecific songs at a long range (i.e., before seeing a mount), suggesting that the other subspecies songs are not considered any greater a threat than allopatric heterospecific songs. Lower responses to other subspecies songs may be the result of reduced recognition of those songs, which likely would create a barrier to dispersal and/or gene flow by making it more difficult for a male with a divergent song to defend a territory acoustically. Contrary to our predictions,

however, males did not respond differently to songs once they approached and had an opportunity to see a red or orange mount. Reduced likelihood of initial approach behavior to songs from the other subspecies was therefore not paired with reduced likelihood of aggression if males had an opportunity to see either a red or orange mount, and presumably visually recognize an intruder. This result is significantly different from the results of the previous playback experiment in this system, in which we concluded that subspecies behave more aggressively to their own subspecies songs using playbacks alone (Greig and Webster 2013). Here, we show that the presence of a visual stimulus (a mount) changed the

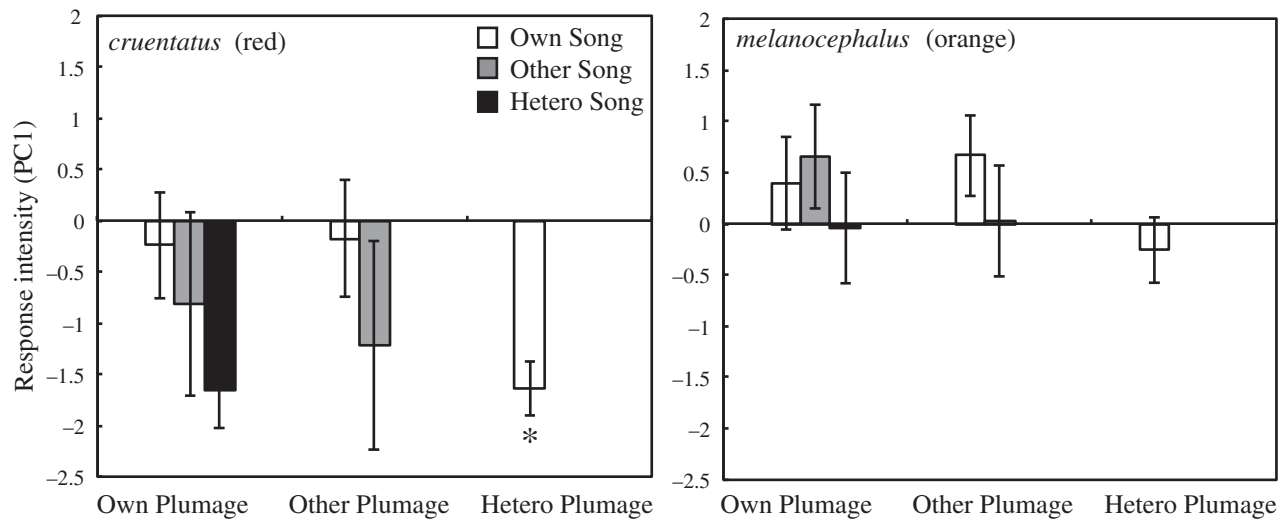


Figure 3. Mean values of response intensity (PC1) for males of each focal subspecies, given that males approached the mount to within 10 m (the same subset of males responding in Fig. 2C and D). Sample sizes are the same as Figure 2C and D for the corresponding treatment types. "Own" refers to song or plumage from the focal birds' own subspecies, "Other" refers to song or plumage from the other subspecies, and "Hetero" refers to white-winged fairy-wren song or plumage. Error bars indicate standard error. Asterisks indicate treatments that were significantly different (detailed in Table 2).

outcome of the playback experiment and caused males to respond with equal aggression to their own and other subspecies songs. Although this aggressive response to mounts paired with other subspecies songs may seem counterintuitive, it may reflect a general tendency of males to attack any visually recognized intruder on their territory, irrespective of that intruder's acoustic signals. This complements previous work showing that failure to be recognized by song actually led to more physical altercations in the long term (Rohwer 1973), which could be the selective mechanism preventing divergent songs from introgressing. Having subspecies-typical songs may allow individuals to moderate territorial interactions by being more easily recognized by neighbors, or by allowing use of acoustic conventions (e.g., song matching) to reduce the need for physical fights (Burt et al. 2001; Temeles 1994). In either case, reduced recognition of song could be detrimental to signalers if they receive equal/greater aggression when recognized visually, and potentially more aggression in the long term because they are less able to moderate conflict acoustically.

Males of both subspecies did not discriminate between red and orange plumage. This lack of discrimination was unambiguous in all measures of response; initial approach was unaffected by mount color, response intensity was equal between red and orange mounts, and inspection/attack behavior given approach to within 10 m was equally likely between red and orange mounts irrespective of song playback. Therefore, male response appears to permit, but not facilitate, the observed introgression of red plumage (i.e., red and orange males should be equally likely to establish and defend territories). We did not find any asymmetries in male

response to plumage that would explain how territorial interactions could facilitate the eastward introgression of red plumage into orange populations. We did not quantitatively compare levels of aggression within trial types between subspecies because there are likely large stochastic differences in aggression levels between populations (e.g., because of differences in density or nesting stage). Nonetheless, red (*cruentatus*) focal males did not appear to be more aggressive than orange (*melanocephalus*) focal males (if anything, the opposite is true; Fig. 3), suggesting that higher aggression in red males does not explain the introgression of plumage traits (as in *Setophaga* warblers; Pearson and Rohwer 2000).

Because previous work indicates that experimentally red-dened males in an orange population of *M. m. melanocephalus* gain higher extra-pair mating success, and because females are known to evaluate extra-pair males at least partly on the basis of plumage color (Karubian 2002; Webster et al. 2008), female preferences, rather than male competitive interactions, likely provide the selective force driving the eastward introgression of red plumage (Baldassarre and Webster 2013). Although we do not have behavioral data on female preferences for red versus orange plumage, our robust exclusion of male territorial response as the driver of the asymmetrical introgression, paired with the pattern of higher extra-pair paternity for red males (Baldassarre and Webster 2013), makes this interpretation the most reasonable working hypothesis for future studies.

Female presence was a significant predictor of male response intensity in *melanocephalus*, such that in trials where

females were present, males were more likely to inspect/attack mounts and had higher response intensity. This may indicate that pairs collaborate in territory defense (Dowling and Webster 2013), or it may indicate that males were engaged in more intense mate guarding behavior when females were present, and our experiments do not allow us to differentiate between these alternatives. The effect of female presence may have been negligible in *cruentatus* because focal birds did not have active nests during our experiment (in contrast to *melanocephalus* pairs, which were actively nesting), which may have dampened any effect of female territory defense or mate guarding on male response.

WHY DO DIFFERENT SIGNAL MODALITIES ELICIT DIFFERENT RESPONSES?

There are several potential reasons for differential male responses to the two signal modalities. First, song is a long-range signal relative to plumage, and if conspecifics benefit by avoiding interactions with divergent phenotypes, then long-range signals should elicit the greatest degree of discrimination because they are the first to be detected and assessed (Uy and Safran 2013). Additionally, males should have the perceptual ability to make fine distinctions between songs, given that acoustic individual/group recognition is an important aspect of fairy-wren social behavior (Payne et al. 1988; Payne et al. 1991). Second, male red-backed fairy-wrens exhibit variable breeding plumages; brown, intermediate, and red-black, and males of all plumage types may defend breeding territories and secure extra-pair copulations (Karubian 2002; Webster et al. 2008; Rowe et al. 2010). Thus, males must engage in territorial interactions with individuals that show considerable variation in plumage, so it may not be advantageous to discriminate finely on the basis of red vs orange plumage if many plumage types are a potential territorial threat.

An alternative explanation for the lack of differential response to plumage is that the differences between red and orange plumage are not great enough for males to perceive them. However, variation in plumage and song between allopatric populations is nonoverlapping for both signal modalities, so there is no quantitative reason to suspect that it is more difficult to differentiate red and orange plumage compared to *cruentatus* and *melanocephalus* song (Fig. S1). Additionally, the previous plumage manipulation experiment showed that plumage color has a strong effect on male mating success (Baldassarre and Webster 2013), indicating that there is some differential behavioral response to red and orange plumage in at least one sex, and implying that the quantitative difference between red and orange is biologically relevant. It is possible that females drive this difference in mating success and have greater visual discrimination ability than males (Bloch 2015), but this would not change our overall interpretation that male behavioral responses permit the introgression of red plumage; it would simply provide a

mechanism for this equal male response (i.e. males may not perceive the plumage difference).

Conclusions

We have found that behavioral responses of territorial males to divergent multimodal signals are consistent with broad geographic patterns of differential introgression. Our results support the hypothesis that singing a subspecies-typical song is advantageous to signalers, because we show that males often ignore other subspecies songs at a long range, which could make it difficult for a signaler with the other subspecies song to defend a territory acoustically. Despite this lower long-range response, signalers with the other subspecies songs do not benefit from reduced aggression once recognized visually, because we show that focal males are equally aggressive to mounts paired with their own subspecies or other subspecies songs once males approach to within a visual range. Our results complement the large body of work indicating that divergent song may hinder male interactions and potentially gene flow between divergent taxa (Baker and Mewaldt 1978; Baker 1991; Searcy et al. 2002; Slabbekoorn and Smith 2002; Patten et al. 2004; Edwards et al. 2005; Podos et al. 2007), and they support a rarely tested mechanism for this disadvantage (namely, reduced recognition at a distance, but maintained aggression during close encounters; Rohwer 1973).

In contrast, equal male response to own and other subspecies plumage appears to permit asymmetrical introgression of that trait, with female preferences likely being the driving force. The lack of male plumage discrimination is in contrast to previous work examining the influence of divergent plumage on behavioral isolation in birds (e.g., Baker and Baker 1990; Patten et al. 2004; Leichty and Grier 2006; Uy et al. 2009). Our study supports the idea that trait divergence is not necessarily the best predictor of reproductive isolation, but that it is instead the behavioral responses of receivers that predict how divergent signals will influence reproductive isolation (Hudson and Price 2014). This complements the large body of work showing the importance of female preferences and receiver sensory biases on speciation (Boughman 2002; Panhuis 2001). Our results differ from previous studies that suggest reduced male aggression to divergent plumage (Vallin et al. 2012), or higher aggression of individuals with foreign plumage (McDonald et al. 2001; Stein and Uy 2006), facilitates hybridization or introgression. These differences may be explained if asymmetrical female preferences for red drives plumage introgression in this system (Baldassarre and Webster 2013), paired with the ease with which plumage and song can be decoupled because males presumably learn songs from within their social group, but may inherit plumage color genes from preferred (red) extra-pair sires (Greig and Webster 2013). The common pattern across this and other studies of differential trait introgression is that

asymmetrical introgression of signals is associated with asymmetrical behavioral responses, even though the specifics of those asymmetrical behaviors vary between systems. Overall, this study suggests that differential responses to divergent signals of multiple modalities may lead to novel combinations of traits upon secondary contact. The extent to which divergent social signals will lead to reproductive isolation and speciation may therefore depend more on the behavioral responses to those signals than on the degree of signal divergence.

ACKNOWLEDGMENTS

We thank Trevor Price, Irby Lovette, Kerry Shaw, Richard Harrison, Jenéle Dowling, Sara Kaiser, Becky Cramer, Scott Taylor, Associate Editor Janette Boughman and two anonymous reviewers for discussion and/or comments on this manuscript. Jordan Karubian, Samantha Lantz, Richard and Jude Luxton, Chad Stachowiak, and Melissa Rowe provided valuable logistical support or assistance in the field. Amanda Corwin and Mateusz Dziekan transcribed videos. Financial support and equipment was provided by the Macaulay Library, the Cornell Lab of Ornithology and the National Science Foundation (M.S.W.). All work was conducted with approval from appropriate animal ethics and permitting agencies (Cornell University Animal Care and Use Committee Approval #0105, James Cook University Ethics Approval #A1340, Scientific Purposes Permit #WISP07773610, Regulation 17 license #SF007698). Emma I. Greig: study conception, data collection/preparation, data analyses, manuscript preparation, and manuscript revision. Daniel T. Baldassarre: study conception, data collection/preparation, and manuscript revision. Michael S. Webster: study conception, manuscript revision.

DATA ACCESSIBILITY

The datasets supporting this article have been uploaded as part of the Supporting Information.

CONFLICT OF INTERESTS

The authors have declared no conflict of interests.

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Associate Editor: J. Boughman
Handling Editor: M. Servedio

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplemental Figure 1. Comparison of plumage hue and song structure between allopatric populations of *M. m. cruentatus* (west) and *M. m. melanocephalus* (east).

Supplemental Table 1. Locations from which playback stimuli were recorded, the number of times each was presented (*n*) and the number of exemplars per site. Excludes heterospecifics.

Supporting Information Data attack proportion intensity

Supporting Information Data response proportion