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Cite this article: Baldassarre DT, Webster MS. 2013 Experimental evidence that extra-pair mating drives asymmetrical introgression of a sexual trait. *Proc R Soc B* 280: 20132175. <http://dx.doi.org/10.1098/rspb.2013.2175>

Received: 20 August 2013

Accepted: 6 September 2013

Subject Areas:

evolution, behaviour, genetics

Keywords:

sexual selection, speciation, asymmetrical introgression, extra-pair mating, plumage colour, sensory bias

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Electronic supplementary material is available at <http://dx.doi.org/10.1098/rspb.2013.2175> or via <http://rspb.royalsocietypublishing.org>.

Experimental evidence that extra-pair mating drives asymmetrical introgression of a sexual trait

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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 versus orange male back plumage colour—but phylogeographic analyses suggest asymmetrical introgression of red plumage into the genetic background of the orange subspecies. We hypothesized that this asymmetrical introgression may be facilitated by sexual selection if red males have a mating advantage over orange males. We tested this hypothesis with correlational data and a plumage manipulation experiment where we 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, we conclude that sexual selection via extra-pair mating is a likely mechanism responsible for the asymmetrical introgression of plumage colour in this system, and is potentially driven by a sensory bias for the red plumage signal.

1. Introduction

Sexual selection is a powerful evolutionary force that can restrict gene flow between diverging taxa upon secondary contact [1–3]. 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 [4,5], and may lead to asymmetrical introgression of traits under positive selection from one population to another. This idea has received theoretical support [6–8], but empirical evidence is limited [9,10]. 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 colour [11,12], has been driven by sexual selection [13,14]. However, the extent to which this phenomenon is more widespread is unclear, and the underlying mechanisms driving such patterns are seldom investigated (but see [15,16]).

The red-backed fairy-wren (*Malurus melanocephalus*) is a small, insectivorous Australian passerine that appears to exhibit asymmetrical introgression of a secondary sexual trait. There are two recognized subspecies that differ primarily in a sexual signal, male nuptial plumage colour [17–19]: the red-backed *M. m. cruentatus* and the orange-backed *M. m. melanocephalus* (hereafter referred to as ‘red’ and ‘orange’; figure 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 [20]. 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 [21–23]. The current continuous distribution of the

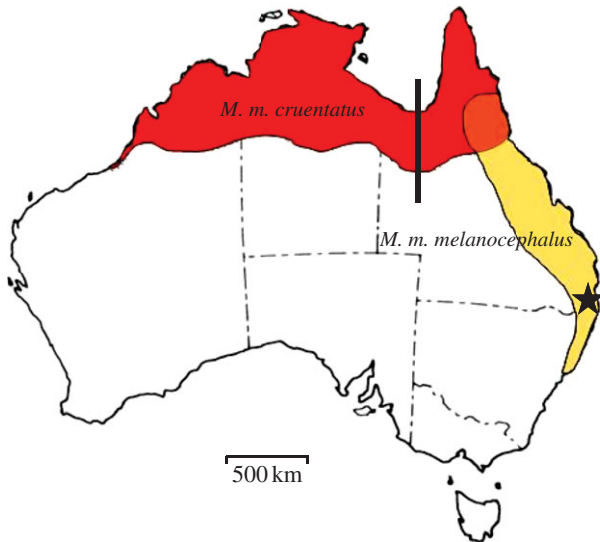


Figure 1. The species range of the red-backed fairy-wren showing the distribution of the two differently coloured subspecies: the red *M. m. cruentatus* in the north, and the orange *M. m. melanocephalus* in the east. The intermediately coloured area in the northeast represents the contact zone between 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.

red-backed fairy-wren across the Carpentarian Barrier probably resulted from secondary contact as the species re-colonized this area after refugial isolation in coastal northern and southeastern Australia [20,21]. 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's song [24]. In stark contrast, the contact zone between plumage types occurs several hundred kilometres to the east of the Carpentarian Barrier, well within the genetic background of the orange *M. m. melanocephalus* subspecies [25,26] (figure 1).

Plumage colour in the red-backed fairy-wren is carotenoid-based [27] 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 [28]. However, in the red-backed fairy-wren, variation in plumage colour is not well explained by environmental variation alone, but instead exhibits a pattern of isolation by distance, suggesting a significant genetic component [26]. In addition, there is a particularly high rate of change in plumage colour across the eastern contact zone, and no significant change across the Carpentarian Barrier [26]. Finally, there is no significant genetic structure across the contact zone between plumage types [20], and no other morphological traits are divergent across the plumage contact zone [26]. 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 colour is subject to sexual selection, as females prefer males in bright nuptial plumage to cryptically coloured males [17–19]. These studies suggest that females assess the back plumage of potential mates, so we hypothesized that sexual selection may be the force driving the introgression of red plumage colour across the hybrid zone.

We tested the prediction that red males have a mating advantage over orange males with correlational analyses and a plumage manipulation experiment in which we 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, we predicted that males with redder (*cruentatus*-like) plumage would have higher reproductive success than more orange (*melanocephalus*-like) males. We 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 [17,18], this component of their reproductive success is thought to be the main factor contributing to the opportunity for sexual selection [29] in this species, as it is for other fairy-wren species [30].

2. Material and methods

(a) Population monitoring and plumage colour 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 northwest of Brisbane, Queensland, Australia, and about 1200 km south of the plumage contact zone (figure 1). We extensively monitored the population for one breeding season (from September 2010 to January 2011) to collect data for our correlational analyses. During this period, we captured, colour-banded and took blood samples from adults, and found all nests in order to band and collect blood samples from nestlings. We collected standard morphometric measurements (e.g. weight, tarsus length, tail length) from all captured birds, and also collected feather samples (six to 12 feathers from the orange back patch) from all males in nuptial plumage. Daily monitoring of individuals allowed us to quantify the date at which males completed their nuptial moult, which is an important indicator of age and reproductive success in a congener, the superb fairy-wren (*Malurus cyaneus*) [31], and may be in the red-backed fairy-wren as well [17]. We were able to classify adult birds into two age classes—second-year and after-second-year—based on degree of skull ossification [32], which was important because, in a different population of red-backed fairy-wrens, older males tend to foray off their territories (probably seeking extra-pair copulations) more often than do younger males (A. L. Potticary *et al.* 2013, unpublished data).

We quantified plumage colour (i.e. coloration of the orange back patch of each male) using reflectance spectrometry of the collected feathers and a mathematical model of the avian visual system as described in [26]. Briefly, we analysed reflectance curves with the program TETRACOLORSPACE [33] using the average avian VS cone-type retina and idealized illumination. We then extracted the angle of the colour 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 colour vector: theta (θ) and phi (ϕ). We focused on theta for our quantification of hue because it best captures the variation from orange to red that characterizes the subspecies [26]; however, the results of the plumage manipulation on phi were qualitatively similar (see electronic supplementary material, figure S3).

(b) Plumage manipulation

We conducted the plumage manipulation experiment during the subsequent breeding season (September 2011–January 2012). Our experimental design consisted of three groups of 13 males each: 'reddened', 'sham' and 'control'. We manipulated the

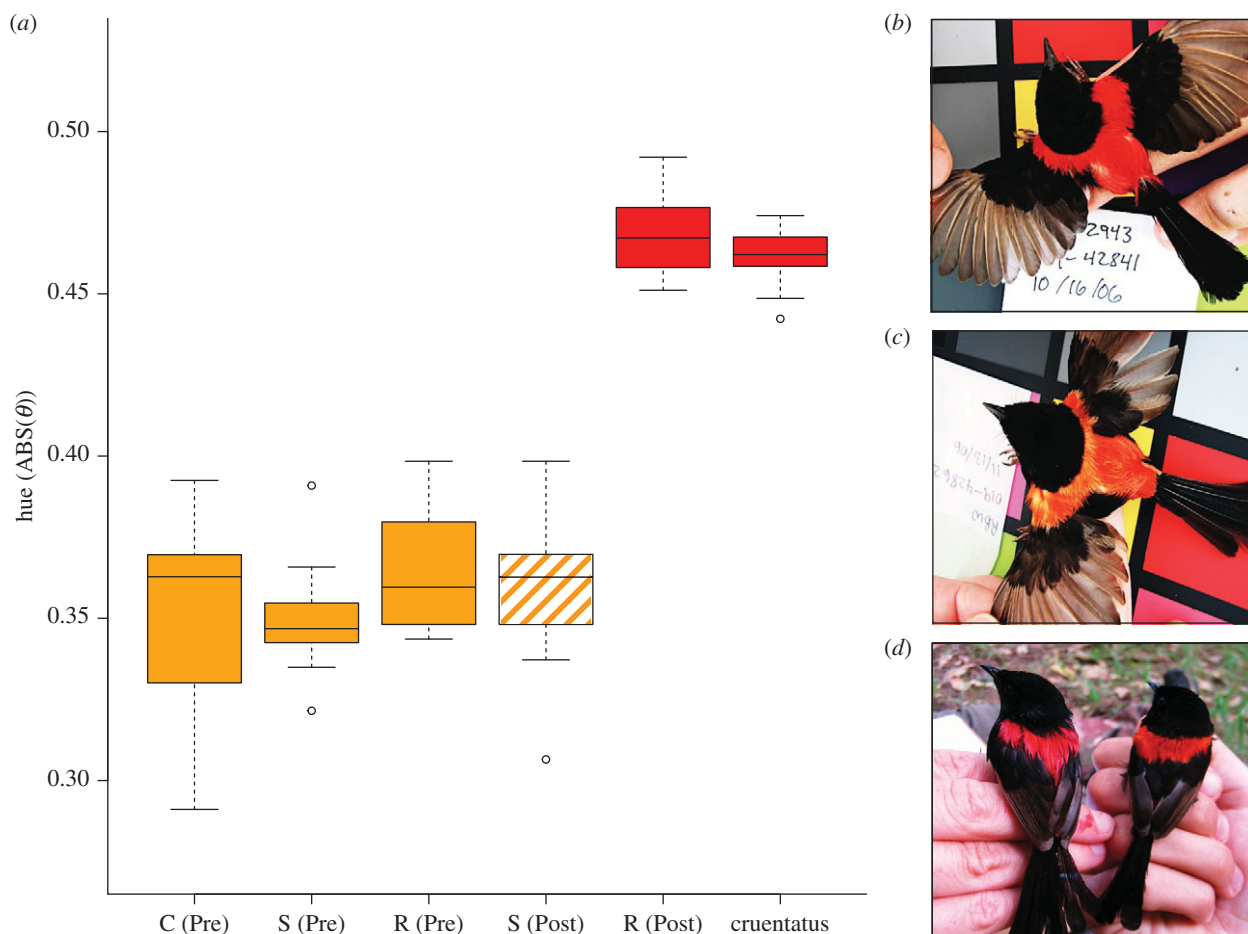


Figure 2. (a) Comparisons of plumage hue between experimental groups (C, control; S, sham; R, reddened) pre- and post-manipulation, and comparison of experimentally reddened males with the natural hue of the red *M. m. cruentatus* subspecies. Values for plumage hue have been converted to absolute values for visualization purposes. (b–d) Photographs of (b) a red *M. m. cruentatus* male, (c) an orange *M. m. melanocephalus* male and (d) comparing a reddened male with a control male.

plumage of reddened males using a non-toxic permanent art marker (Prismacolor Carmine Red) and applied a colourless marker (Prismacolor Colourless Blender) to the sham males. We 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. To analyse the effect of these manipulations on plumage colour, we used paired-sample *t*-tests to compare the colour before and after manipulation. The colourless marker used in the sham manipulation did not significantly alter plumage hue, saturation or brightness (all *t* between -1.4 and -0.5 , d.f. = 12, all $p > 0.1$). The red marker did not alter saturation ($t = -1.57$, d.f. = 12, $p = 0.15$) or brightness ($t = 1.85$, d.f. = 12, $p = 0.09$), but it significantly reddened the hue of males ($t = -19.8$, d.f. = 12, $p < 0.001$; figure 2), 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 ($t = 1.5$, d.f. = 22.9, $p = 0.16$; figure 2). See electronic supplementary material, figures S1–S3 for further details.

At the beginning of the breeding season—after males had completed their pre-nuptial moult, secured a territory and formed social pairs, but before we observed any nesting behaviour—we 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. We 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 colour as measured by hue (figure 2), saturation or brightness (all ANOVA $F_{2,36} < 2$, all $p > 0.1$). Experimental groups were also similar in other

potentially important characteristics (see electronic supplementary material for details). After manipulation, we released all experimental males back into the population and monitored all subsequent nesting attempts.

(c) Genetic paternity analyses

We genotyped all individuals at seven highly polymorphic microsatellite loci and assigned paternity using the program CERVUS v. 3.0 [34] (see electronic supplementary material, table S1 for details). The paternity analyses revealed a high overall rate 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 the 2011 and 2012 breeding seasons, respectively.

Using these paternity results, for each male, we 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). See electronic supplementary material, table S2 for how paternity was distributed across experimental groups.

(d) Statistical analyses

For non-normally distributed response variables, we analysed the data using either Poisson or binomial distributions, or non-parametric mean comparisons. Using the correlational data, we

Table 1. Results of six generalized linear models of the effect of naturally varying plumage hue on various measures of reproductive success.

model	<i>n</i>	estimate	s.e.	<i>t</i>	<i>p</i>
no. within-pair young	57	−10.589	9.683	−1.092	0.275
no. extra-pair young	57	−5.59	7.693	−0.727	0.467
no. 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 ≥ one extra-pair young	57	−0.542	14.913	−0.036	0.971
cuckolded	57	0.875	21.311	0.041	0.967

analysed the effect of plumage colour on reproductive success seven different ways. First, we fitted 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, we used a Poisson distribution with a log link function. We 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). We 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, we conducted a pair-wise analysis of the plumage hue of a cuckolded male compared with the plumage hue of the male that cuckolded him using a paired *t*-test. We analysed 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 pair-wise comparisons using Wilcoxon rank-sum tests. All statistical analyses were performed using R v. 2.15.3 [35].

3. Results

(a) Natural plumage hue and reproductive success

We 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 1). In addition, there was no significant difference in plumage hue between males that lost paternity and the males that cuckolded them (paired *t*-test, $t = -0.17$, d.f. = 19, $p = 0.87$).

(b) 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 $H = 3.7$, d.f. = 2, $p = 0.16$; figure 3a), but there was a significant difference in number of extra-pair young ($H = 8.72$, d.f. = 2, $p = 0.01$; figure 3b), which resulted in a significant difference in total reproductive success ($H = 7.27$, d.f. = 2, $p = 0.03$; figure 3c). 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 ($W = 31$, $n_{1,2} = 13$,

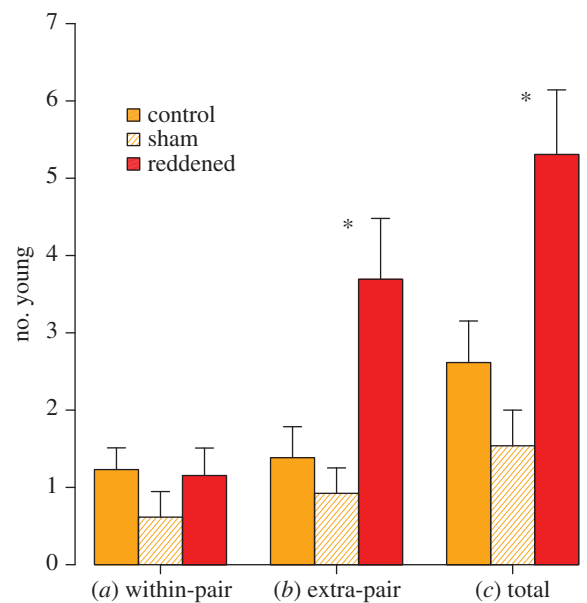


Figure 3. The resulting effects of plumage manipulation on three components of reproductive success: (a) number of within-pair young, (b) number of extra-pair young and (c) total number of young. Error bars represent standard errors, and asterisks separate values that are significantly different.

$p = 0.003$) 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 ($W = 35.5$, $n_{1,2} = 13$, $p = 0.01$). To further explore the effect of introducing reddened males into the population, we analysed the population-wide effect of plumage hue on number of extra-pair young, including all naturally varying orange males as well as reddened males, using a generalized linear model as above, and found a significant positive effect of plumage hue ($n = 59$, estimate = 6.6, s.e. = 1.5, $t = 4.35$, $p < 0.001$; see electronic supplementary material, figure S4). Plumage manipulation had no effect on the proportion of males cuckolded (Kruskal–Wallis $H = 2.91$, d.f. = 2, $p = 0.23$) or cuckoldry rate ($H = 4.62$, d.f. = 2, $p = 0.1$).

4. Discussion

(a) Asymmetrical introgression via extra-pair mating

We found support for the hypothesis that sexual selection via extra-pair mating is driving the observed asymmetrical introgression of red back plumage from the *M. m. cruentatus* subspecies into the genetic background of the orange

M. m. melanocephalus subspecies. 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. 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 [17,18], 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 [30]), and the evolutionary force driving asymmetrical introgression of this sexual signal.

Behaviourally, there is evidence that females have the opportunity to assess potential extra-pair mates. Males foraging onto a neighbouring male's territory exhibit 'petal carrying', in which they display flower petals to potential extra-pair females [25,36]. Foraging males also display their back plumage to females in a stereotyped 'puff-back display' [25]. We 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 we did not quantify any behavioural 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 [37], among other possibilities. Several studies have shown that different male traits contribute to within- and extra-pair reproductive success [38–40]. 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 probably have ample opportunity to evaluate within-pair mates, but may be under significant temporal constraint when assessing potential extra-pair mates owing to mate guarding and the brevity of territorial intrusions (A. L. Potticary *et al.* 2013, unpublished data). Under these circumstances, plumage colour 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 colour 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 [41]. However, plumage colour is probably not a consistent and reliable signal during pair formation, as this typically occurs before males have completed their pre-nuptial moult (D. T. Baldassarre 2013, unpublished data). 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.

(b) Sensory bias in females

We found no support for the hypothesis that naturally redder males have higher reproductive success than more orange males in this population. Because the study population is located in an allopatric area of the orange subspecies, there is little variation in plumage colour compared with populations within the

plumage contact zone (Brisbane hue s.d. = 0.02 versus 0.04 in contact zone; see [26]). Only when experimentally manipulating the plumage of males outside the natural range of hue found in this population did we detect an effect. This suggests that *M. m. melanocephalus* females may have a latent preference (i.e. sensory bias [42]) 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 [43] or because of a specific bias towards red is unclear. We 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 (we were 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 [44]). 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 [14].

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 [16]. We did not quantify any behavioural differences between experimental groups in this study, but a separate mount presentation experiment suggests that territorial males do not differ in their aggressive responses to red versus orange mounts (E. I. Greig *et al.* 2013, unpublished data). Finally, red plumage may be an honest indicator of quality in this system, but we suggest that the lack of selection on naturally varying plumage hue is inconsistent with this hypothesis, and we did not find any association between male condition and hue (E. I. Greig *et al.* 2013, unpublished data).

(c) 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 [7,8]. Our 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. The extent of gene flow resulting from such introgression in this system is unclear, and a more extensive multilocus geographical cline analysis of the hybrid zone is currently under way. 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 [24]. Our 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 [45].

5. Conclusion

In summary, we found support for the hypothesis that asymmetrical introgression of a sexual signal—red back plumage colour—is facilitated by sexual selection in the red-backed fairy-wren. This introgression is likely to be mediated solely by extra-pair mating behaviour, as we did not detect an effect of plumage manipulation on within-pair reproductive success. Because we did not find any effect of naturally varying plumage colour on reproductive success in the orange population, this probably 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.

All research activities involving live birds were approved by the Animal Ethics Committee at James Cook University, Queensland, Australia, and also by the Institutional Animal Care and Use Committee at Cornell University.

Acknowledgements. Thank you to Webster and Lovette laboratory members for input and comments. Seqwater allowed us access to the field site. Roger McNeill and the staff at Queensland University of Technology's Samford Ecological Research Facility provided logistical support in the field. Scores of field assistants helped greatly in data collection. Data supporting this paper are available upon request.

Funding statement. Research was supported by a National Science Foundation (USA) grant to M.S.W. as well as grants awarded to D.T.B. from the Cornell University Department of Neurobiology and Behavior, Cornell Lab of Ornithology and Sigma Xi. D.T.B. was supported by the Cornell Lab of Ornithology during part of this research.

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