Opposing dominance within a color gene block is associated with maladaptive hybrid signal discordance

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Abstract

Color divergence is increasingly recognized as important for speciation in songbirds through its influence on social dynamics. However, the behavioral mechanisms underlying the eco-evolutionary feedback that acts across species boundaries is poorly understood. The hybrid zone between *Setophaga occidentalis* (SOCC) and *S. townsendi* (STOW) in the Cascade mountain ranges provides a natural observatory to test the interplay between genetics and social behaviour in maintaining species boundaries. Recently, we found that selection within a gene block underpinning color variation (ASIP-RALY) has maintained a stable and narrow hybrid zone. Here we investigated the social signaling roles of cheek darkness and flank streaking, two color traits linked to ASIP-RALY that reflect opposing dominance of SOCC and STOW alleles. We found that both traits act as honest badges of status, as they predicted male breeding quality. The opposing dominance effects of ASIP-RALY resulted in signal discordance for heterozygotes, which in turn was associated with inferior hybrid territorial performance, a fitness proxy quantified by vocal and physical responses of resident males to a decoy intruder. Taken together, this study highlights a potential behavioral mechanism underlying selection acting on a simple genetic architecture that has maintained a stable species boundary over decades despite significant gene flow.

Keywords: speciation, ASIP, RALY, color genes, badge of status, hybrid fitness, honest signal, territoriality, *Setophaga*.

Introduction

Bird plumage is often characterized by distinct patches of pigments (e.g., carotenoids, melanins) where the patch size or color intensity can relay information about an individual's genetic or phenotypic quality, including 'good genes', nutritional state, immunocompetence, parental investment, or territorial dominance (McGraw and Ardia 2003; Hill 2006; Searcy and Nowicki 2010; Svensson and Wong 2011). In the context of a social environment, 'badges of status' may signal social dominance as a mechanism to limit costly combats among territorial individuals (Rohwer 1975; Studd and Robertson 1985). Social dominance can be costly due to detrimental levels of testosterone along with frequent energy- and time-demanding territorial challenges (Goymann and Wingfield 2004). Therefore, badges of status are thought to emerge and be maintained in populations characterized by individuals that vary in their ability to overcome these costs (i.e., body condition, aggression), as well as repeated territorial contests for mates and limited resources (1,2). To be considered an 'honest' signal, plumage badges should be variable among counterparts of the same sex, correlate with quality or combat ability of the carrier (Rohwer 1975, 1982; Rohwer and Rohwer 1978), and be a component of aggressive interactions among counterparts, either by dissuading combat among individuals of different status or triggering combats between individuals with similar badges (Rohwer 1975). Support for honest badges of status is often context dependent, but there is ample observational and experimental evidence to suggest that this mechanism of territorial signaling is widespread (e.g., (Studd and Robertson 1985; Møller 1987; Safran et al. 2008; Hund et al. 2020).

Badges of status may be divergent among closely-related species (Rohwer 1982) and thus a potentially important component of speciation. When badges established in isolated populations come into secondary contact, divergent systems may lead to signal interference and compromised territorial performance among hybrid individuals with discordant badges (Birch 1957; Schultz and Switzer 2001; Gröning and Hochkirch 2008). Incompatible badges of status within hybrids may thus incur reduced hybrid fitness. Badges of status and their genetic basis are often investigated within species or populations, but rarely across species boundaries. Here we assessed multiple badges of status within allopatric and hybrid zone populations of two closely related wood-warbler species, *Setophaga townsendi* and *Setophaga occidentalis*, to understand the potential role of plumage traits and social dominance in speciation.

Divergent badges of status may be involved in speciation between *S. townsendi* (hereafter abbreviated as 'STOW') and *S. occidentalis* ('SOCC') due to frequent heterospecific male competitions (Pearson and Rohwer 2000) within the narrow hybrid zone along the Cascade mountain range (Rohwer and Wood 1998; Wang et al. 2019, 2020*b*). Recent evidence revealed that the key genomic differentiation between allopatric parental populations resides within a color gene block, ASIP-RALY, which pleiotropically underpins variation in multiple color traits among hybrids sampled in the hybrid zone; particularly the extent of black pigmentation (melanin) and yellow (carotenoid) coloration in the crown, cheek, breast, and flank, all of which are diagnostic for the two species (Wang et al. 2020*b*). Spatiotemporal analysis of ASIP-RALY allele frequency revealed selection acting on this gene block (Wang et al. 2020*b*), although the mechanism of selection remained unclear.

One potential mechanism underlying selection against individuals heterozygous for ASIP-RALY genotypes could be reduced hybrid fitness through incompatible badges of status, or the breakdown of signal reliability (Vortman et al. 2011, 2013). Species-specific carotenoid and melanic color features likely serve as badges of status in this system, as they vary in intensity within species (Jackson et al. 1992;

Wang et al. 2020*b*) and are involved in male-male competition (Pearson 2000; Pearson et al. 2000). Two of these ASIP-RALY-associated traits, flank streaking and cheek darkening, demonstrated opposing dominance: the STOW allele is dominant in flank streaking, whereas the SOCC allele is dominant in cheek darkening (Wang et al. 2020*b*). Based on this pleiotropic opposing dominance effect of ASIP-RALY gene block, we predicted discordant cheek and flank ancestry in heterozygous individuals. If cheek darkening and flank streaking act as honest signals within species and are divergent between species, individuals with discordant badges of status may disproportionately suffer the costs of territorial disputes, resulting in reduced social dominance and fitness.

We investigated the mechanism underlying selection on the ASIP-RALY gene block that may maintain or reinforce the STOW-SOCC species boundary by addressing three questions:
(1) Do cheek and flank plumage patches act as badges of status within species and diverge between species?
(2) Does the pleiotropic opposing dominance effect of ASIP-RALY lead to discordance of badges of status in the hybrid zone?
(3) Are hybrid individuals with discordant badges of status characterized by reduced territorial performance?

Materials and Methods

Study site

We carried out this study in the hybrid zone within the Cascades mountain range of Washington as described in our previous study (Wang et al. 2019). Detailed sampling information is summarized in the dryad spreadsheets (https://doi.org/10.5061/dryad.j6q573nd2).

Breeding male status

We sampled breeding males in allopatric populations of STOW (N = 126) and SOCC (N = 7), as well as the hybrid zone (N = 273) during the 2015 and 2016 breeding seasons. To infer male breeding quality (size and breeding status), we measured a range of indices including body weight, cloacal protuberance (CP), tarsus length, wing chord length, and bill length. We used principal component (PC) analysis (centered and scaled) with *prcomp* in R and considered PC1 to represent breeding condition (Table S1). Among the 406 males, we performed imputation to estimate missing values of each variable (n = 11 for body weight, 2 for CP, 2 for tarsus length, 1 for wing chord, and 0 for bill length). First, we calculated a distance matrix for the condition indices with the *dist* function in R. For each individual missing variable, the value of the variable from the nearest neighbor was adopted as the imputation entry.

Plumage coloration

We used measurements of cheek coloration and flank streaking from our previous study (Wang et al. 2020*b*). Briefly, for the cheek colour of breeding males, we quantified the proportion of yellow relative to black pigmentation in LAB colour space using Photoshop (Adobe Photoshop CS 2004). Mid and lower flank streaking were scored and summed to quantify total flank streaking (Wang et al. 2020*b*). Age correction was conducted for each of the plumage traits to control for age-related pigmentation variation. Briefly, an age-correction factor was calculated as the within-quartile difference between median pigment values of each age class. We then adjusted the age-class-specific differences at each quartile of the pigment measurement with the age-correction factor (Wang et al. 2019, 2020*b*).

Badges of status

To test whether flank streaking and cheek coloration may act as badges of status or signal individual quality, we conducted linear regression of breeding condition PC1 against each of the plumage variables accounting for population (parental STOW, SOCC, or hybrid zone):

$$\text{lm}\,(\text{condition} = \text{plumage} \times \text{population}) \qquad [1]$$

A significant slope would indicate that these plumage traits are associated with individual condition within populations and therefore have the potential to act as badges of status. A significant interaction term would indicate divergence in signal honesty among populations.

Color genotype

Genotype data was acquired from our previous study (Wang et al. 2020*b*), in which the genotypes of color gene block ASIP-RALY was denoted as OO, OT, TT for homozygous SOCC, heterozygous, and homozygous STOW, respectively. Dominance coefficients for each plumage trait were determined by the following equation:

$$h = (\bar{Z}_{TT} - \bar{Z}_{OT})/(\bar{Z}_{TT} - \bar{Z}_{OO}) \qquad [2]$$

where $\bar{Z}_{OO}$, $\bar{Z}_{OT}$, and $\bar{Z}_{TT}$ are mean phenotype individuals with OO, OT, and TT ASIP-RALY genotype. The STOW allele is dominant if $h = 0$, whereas the SOCC allele is dominant if $h = 1$. The bootstrap distribution of h for each trait was generated by resampling individuals with replacement 10,000 times and calculating h each time. The dominance coefficients for flank streaking and cheek darkening were denoted as h_{flank} and h_{cheek}, respectively.

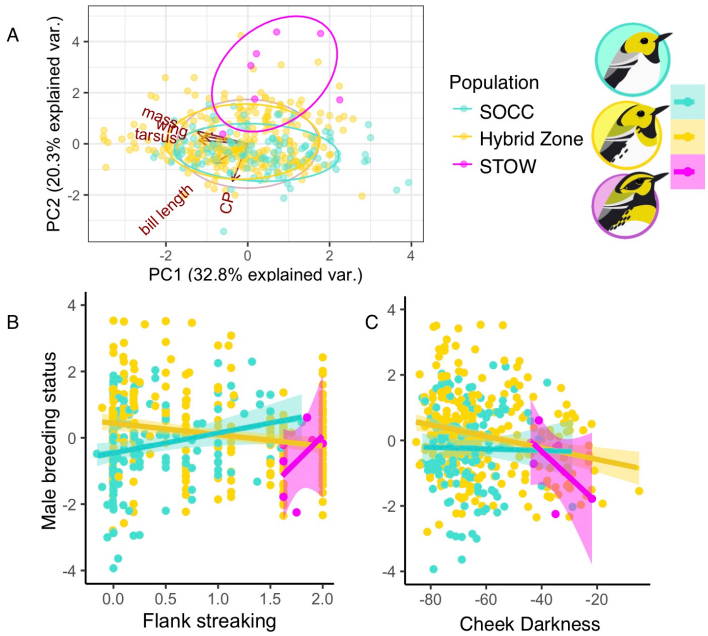

Fig. 1. Male breeding condition signals: flank streaking and cheek coloration in the hybrid zone and parental populations (SOCC and STOW). A, Male breeding quality PCA in which -PC1 was employed as a breeding quality index. **B**, Flank streaking significantly predicts breeding quality of males differently among populations. **C**, Cheek darkness also predicts male breeding conditions, but the association was not significantly different among populations.

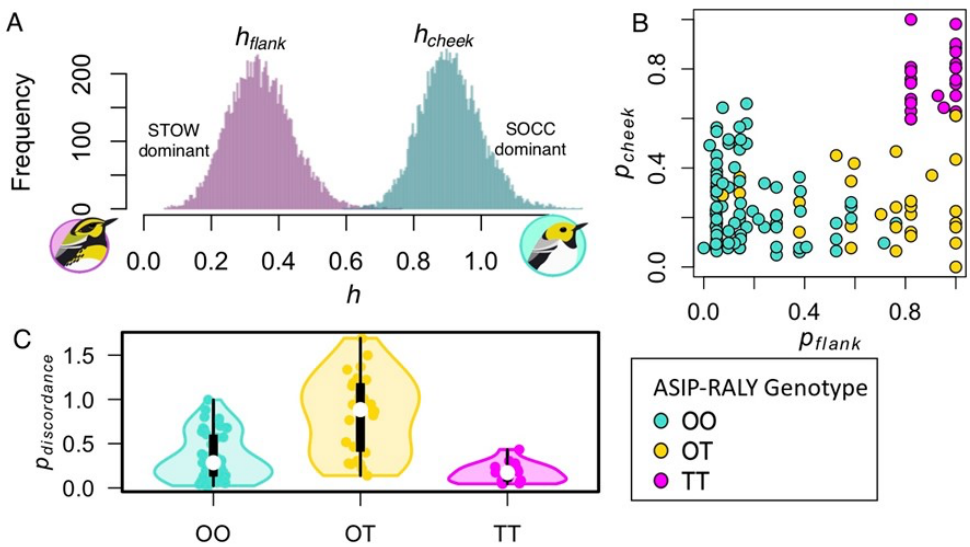

Fig. 2. Opposing dominance in ASIP-RALY gene block leads to plumage ancestry discordance in hybrids. **A**, The distribution of dominance coefficients of flank streaking (left) and cheek darkening (right), where the STOW variant is dominant for flank streaking whereas the SOCC variant is dominant for cheek darkening (Wang et al. 2020*b*). **B**, Ancestry indices of cheek darkening (p_{cheek}) and flank streaking (p_{flank}) of individuals with ASIP-RALY genotypes: homozygous SOCC (OO), heterozygotes OT, and homozygous STOW (TT) genotype. **C**, Greater plumage ancestry discordance in individuals with OT than OO or TT.

Territorial performance: aggression intruder assay

To assess territorial performance of breeding hybrid males that were genotyped and had their plumage traits scored (n = 59), we quantified territorial defense of the resident males in terms of vocal (song) and physical (flight attacks) responses during an artificial territorial intrusion. We revisited territories 5–10 days after banding to carry out the territorial intrusion trials. Two researchers, one observer and one taking notes, stood 15 m from the decoy (see below). An initial quiet period of 3 min was performed to control for disturbance around the area when setting up the experiment, and then a playback consisting of a series of song recordings were played during the following 12 minutes. The response of focal males was recorded in terms of three variables: (1) song rate, (2) short-distance flight attacks (flight around the decoy within 5 meters), and (3) long-distance flight attacks (flight around the decoy between 5 to 15 meters). Clay decoys were painted as mature individuals (> 2 years) in breeding plumage (Figure 3A) of STOW, SOCC, and hybrid (with hybrid index = 0.5) following Rowher & Wood (1998). At each territorial intrusion trial, a decoy was randomly selected as visual stimulus. Decoys were presented on a tree branch that was about two meters above the ground, within 5 meters of vegetation on which the responding male could alight without being forced to contact the mount. Six songs (recorded with Marantz PMD660 and an Audio-Technica 815a Shotgun microphone), each lasting two minutes, were played with a small speaker (Jam® HX-P250) placed 1 meter below the mount. The songs were recorded from different populations, with two songs from each of the *S. townsendi* allopatric populations, the hybrid zone, and the allopatric *S. occidentalis* zone. To be consistent with behavior scoring, a single observer (SW) described all behavior trials.

Plumage ancestry

The age-corrected cheek and flank streaking were scaled from 0 to 1 with the following transformation function:

$$x' = \frac{x - x_{min}}{x_{max} - x_{min}} \quad [3]$$

where x is the plumage (cheek coloration or flank streaking) estimate of each individual. To infer hybrid ancestry, we calculated hybrid plumage ancestry (p) with the following equation:

$$p = \frac{x' - \bar{x}_{SOCC}}{\bar{x}_{STOW} - \bar{x}_{SOCC}} \quad [4]$$

$\bar{x}_{SOCC}$ and $\bar{x}_{STOW}$ are the estimated means of plumage variation in SOCC and STOW populations. The ancestry discordance ($p_{discordance}$) is the absolute difference of the ancestry indices of cheek coloration (p_{cheek}) and flank streaking (p_{flank}):

$$p_{discordance} = |p_{cheek} - p_{flank}| \quad [5]$$

All analyses were conducted in R version 3.6.3 (R Core Team 2017).

Results

Badges of status

Cheek coloration and flank streaking predicted male breeding quality differently among populations. Breeding quality PC1 explained 32.85% of the variation in body condition and was negatively associated with all of the composite variables (Figure 1A). Thus, we used -PC1 as the breeding quality index, so that greater positive values correspond to greater male size, mass, and breeding status. Flank streaking and breeding quality were significantly and positively associated ($F_{(5, 397)}$ = 5.32, p = 9.68 × 10^{-5}; Figure 1B), yet this association was divergent among populations as there was a significant interaction between flank streaking and population ($F_{(2, 397)}$ = 6.76, p = 0.001). Breeding quality was also significantly predicted by cheek coloration ($F_{(5, 398)}$ = 4.93., p = 0.0002; Figure 1 C), but this association was not significantly divergent among populations ($F_{(2, 399)}$ = 1.56, p = 0.212).

Pleiotropic opposing dominance and $p_{discordance}$

Flank streaking and cheek darkness demonstrated opposite dominance, such that the O allele is dominant for cheek darkening, while the T allele is dominant for flank streaking (Wang et al. 2020b) (Figure

2A). This resulted in sickle-shaped associations between cheek darkening and flank streaking (Figure 2B) within the hybrid zone, where heterozygotes (OT) had cheek darkening similar to OO individuals, but TT-like flank streaking. As a result, we found a significant association between $p_{discordance}$ and ASIP-RALY genotypes among hybrids (Kruskal-Wallis rank sum test, $\chi^2_{(df=2)} = 35.15$, $p = 2.3\times 10^{-8}$; Figure 2C). There was greater $p_{discordance}$ in heterozygous (OT) than OO (Wilcoxon rank sum test with Bonferroni correction, $p = 8.0 \times 10^{-6}$) and TT (Wilcoxon rank sum test with Bonferroni correction, $p = 3.8 \times 10^{-7}$), while homozygotes were not significantly different ($p > 0.05$) (Figure 2C).

Table S1 Loadings of each male breeding quality variable in each PC.

	PC1	PC2	PC3	PC4	PC5
mass	-0.571	0.293	-0.119	-0.124	-0.747
tarsus	-0.534	0.080	0.297	-0.613	0.494
wing	-0.482	0.171	-0.517	0.536	0.428
CP	-0.167	-0.835	-0.452	-0.251	-0.086
bill length	-0.359	-0.425	0.653	0.508	-0.081

Territorial defense

Hybrid males with greater $p_{discordance}$ demonstrated inferior territorial performance. The strength of territorial defense of the resident males was estimated by the physical attacks represented by PC1 (Table S1) for flight attack observations (short and long-distance flights) and song responses during the territorial intrusion trials. Flight PC1 was positively associated with greater flight attacks and explains 64.92% of the variation of flight attacks among males. Males with greater $p_{discordance}$ exhibited reduced flight attacks (Spearman's $\rho = -0.38$, $p = 0.037$; Figure 3B) and song rates in response to an artificial intruder (Spearman's $\rho = -0.47$, $p = 0.01$; Figure 3C). To visualize the adaptive landscape with respect to p_{cheek} and p_{flank}, we approximated male fitness by territorial performance, which is the averaged flight PC1 and song response to artificial intruder (Figure 3D). To ensure that flight attacks and song responses contributed to territorial performance equally, we scaled the two variables with eq 3 before averaging. The bottom right corner of signal discordance (Figure 3D) corresponds to the valley of territorial performance, while the ridge of performance occurs along the diagonal space of signal concordance.

Discussion

We found that two plumage traits, cheek melanism and flank streaking, are linked to breeding quality in SOCC and STWO, which suggests that they may act as honest signals of quality among male conspecifics or potential mates within a social environment. Additionally, we demonstrated a pleiotropic opposing dominance effect of the ASIP-RALY gene block which led to discordant badges of status within the hybrid zone. Hybrids that exhibited greater discordance among badges were, in turn, associated with compromised territorial performance (Figure 3D) represented by reduced attack and song rate in response to an intruder. Thus, we highlight a potential behavioral mechanism contributing to selection against hybrids and the maintenance of a species boundary between SOCC and STWO.

Badges of status

Both cheek darkening and flank streaking predicted male breeding quality, indicating that these phenotypes have the potential to function as honest badges of status in territorial competition or mate attraction for SOCC and STOW. Sexually dimorphic carotenoid and melanin-based plumage traits have been implicated as badges of status in many species (Rohwer 1975; Studd and Robertson 1985; Senar 2006; Blount and McGraw 2008; Searcy and Nowicki 2010; Chaine et al. 2018). Carotenoids are red or yellow pigments that are exogenously acquired from food or biochemically converted (Hill 2000; Svensson and Wong 2011). More vibrant colours therefore indicate greater resource acquisition and have been hypothesized as a signal of individual quality in males, indicating greater body condition, immunocompetence, 'good genes', and particularly the ability to secure and defend resource-rich territories (McGraw and Ardia 2003; Hill 2006; Blount and McGraw 2008). In contrast, melanins in black or brown patches are an expression of melanocortin systems, where melanogenesis is closely tied to intrinsic endocrinal pathways such as testosterone production (McGraw et al. 2002; Bókony et al. 2008; Galván and Solano 2016). As a result, males with darker melanistic plumage traits have been associated with greater territoriality, aggressiveness, and social dominance (Roulin 2016; San-Jose and Roulin 2018). Evidence for plumage traits as social signals is widespread, yet the evolution and maintenance of signal honesty is likely dependent on genetic, environmental, and/or social conditions (Senar 2006; McGraw 2008; Safran et al. 2008; Tibbetts and Safran 2009; Hund et al. 2020). Brighter yellow cheeks and greater flank streaking were associated with greater breeding quality (i.e., larger size, greater body condition) with different strengths (slopes in Figure 1). Given that size traits and body condition metrics are often associated with individual quality or social dominance (Smith and Parker 1976; Alatalo and Moreno 1987), this suggests that: (1) plumage traits can reliably signal individual quality and dominance in both species, but (2) different signals are operating within each species.

Table S2 Loadings of each flight attack variable in the territorial intrusion trials.

	PC1 (64%)	PC2 (36%)
Short flight	0.734	0.679
Long flight	0.679	-0.734

Opposing dominance of ASIP-RALY and signal discordance

Opposing dominance within the ASIP-RALY gene block (Wang et al. 2020*b*) resulted in discordance between cheek and flank badges of status within hybrids, which was associated with compromised territorial performance (Figure 3). The effect of this opposing dominance in ASIP-RALY is similar to the previously documented opposing dominance effect of two loci in *Mitoura* butterflies (Forister 2005), where the F1 hybrids (heterozygous for both loci underlying host plant preference and performance) demonstrated preference for cedar but better performance in cypress habitat. While the habitat preference and performance traits are co-adaptive within *Mitoura* species, discordance of preference and performance led to poor hybrid fitness (Forister 2005). In *Setophaga* warblers, opposing dominance occurred within one genetic region as opposed to two, which resulted in maladaptive signal discordance in hybrids (Figure 3D). This observation is consistent with a single-locus-two-alleles model of speciation (Moore 1979), although there are other possible genomic contributors of speciation in this *Setophaga* system (Wang et al. 2020*a*).

Discordant signals have the potential to disrupt hybrid interactions such as male-male competition or mate attraction. Unreliable badges could lead to more frequent costly battles with aggressive, socially dominant males, potentially requiring greater investment in survival and less in the acquisition and defense of high-quality territories and mates (Møller 1987; Qvarnstrom 1997). Therefore, such an effect could contribute to the selection against hybrids that maintains a stable

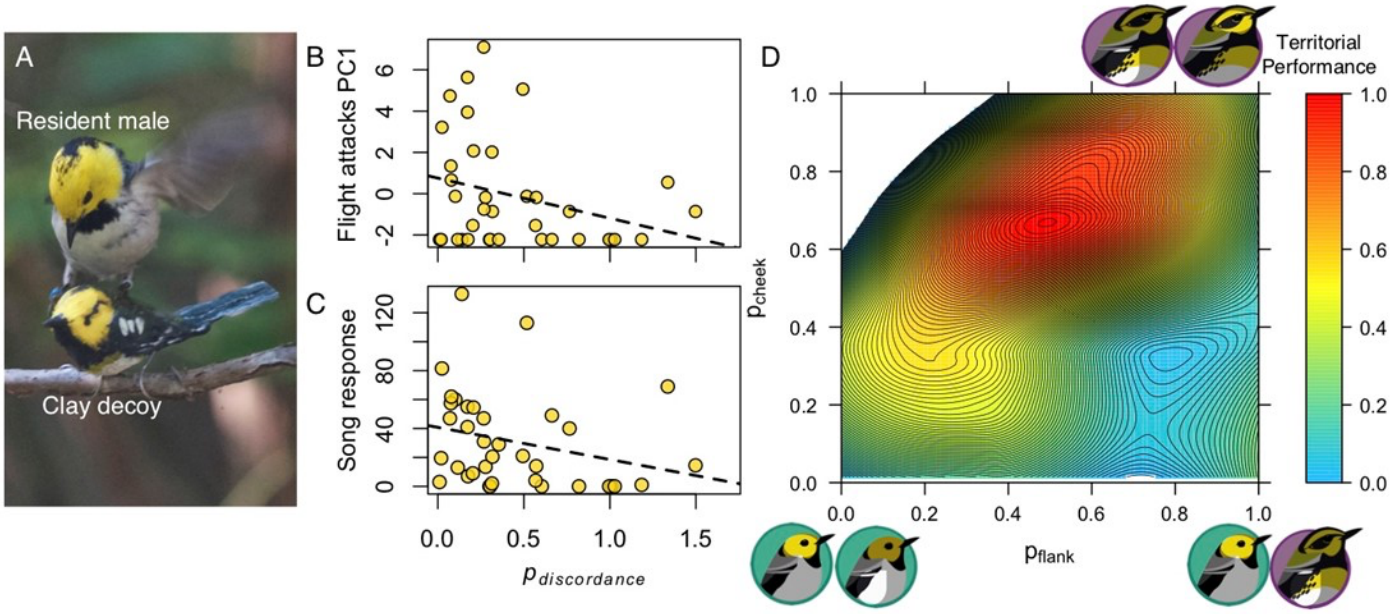

Fig. 3. Reduced intruder territorial defense of hybrid males with greater ancestry discordance ($p_{discordance}$) of plumage badges. A, Short flight attack of the focal male during the intruder stimulation trial (photo credit: Else Mikklesen). **B-C**, Males with greater $p_{discordance}$ demonstrate reduced flight attacks (**B**, rho = -0.47, p = 0.037) and reduced song responses (**C**, rho = -0.38, p = 0.010). **D**, Territorial performance with respect to the p_{flank} and p_{cheek} in the hybrid zone, where the males with STOW-like flanks and SOCC-like cheeks demonstrated the lowest territorial performance.

species boundary (Wang et al. 2020*b*). In line with this hypothesis, we found that the extent of signal discordance was associated with reduced hybrid territorial performance (Figure 3D). Territoriality is an important proxy of breeding male fitness both within and between SOCC and STOW (Pearson and Rohwer 2000; Townsend et al. 2000). For migratory songbirds like *Setophaga* warblers, males arrive at the breeding grounds before females to acquire high-quality territories and attract mates, such that the ability to defend territories is essential to reproductive success and male fitness (Morbey and Ydenberg 2001). Our results show that hybrid males with discordant badges of status exhibited less aggressive or defensive behaviors in response to an artificial intruder (i.e., flight attacks, song rate), implying reduced fitness for these individuals.

This is consistent with the hybrid inferiority or parental-fitness asymmetry hypotheses, both of which posit that hybrids display inferior fitness in some way relative to their parents which in turn can maintain a narrow and stable hybrid zone (Barton and Hewitt 1985, 1989; Rohwer and Wood 1998; Wang et al. 2019, 2020*b*). While we assume that territorial aggression is associated with fitness in this case, we could not assess breeding success or fecundity to directly link hybrid fitness with the level of signal discordance. Poor hybrid fitness may also manifest itself in other ways that we did not measure, such as reduced parental investment or survival, which could similarly limit lifetime fitness and reinforce species boundaries (Servedio and Noor 2003). While these factors deserve additional study to complete our understanding of *Setophaga* hybrid fitness, we highlight that territorial dynamics and altered social interactions in general are an intriguing mechanism by which the species boundary could be maintained.

Aggression signals & speciation

We provide evidence for the role of male-male competition signals in speciation. Despite the long-standing view that sexual selection contributes to the evolution of reproductive isolation, signals such as plumage traits underlying male-male competition have been overlooked until recently (Polechová and Barton 2005; Pfennig and Pfennig 2010; Tinghitella et al. 2018). The opposing dominance of ASIP-RALY leading to maladaptive discordance of hybrid badges of status is consistent with accumulating evidence that both male competition and mate choice signals are involved in reproductive isolation. Importantly, our results do not preclude that cheek and flank plumage traits may also play a role in mate choice. Recent evidence highlights the potential for some plumage traits to have dual, interacting functions, co-evolving to signal multiple receivers (Tarof et al. 2005, Senar 2006, Karubian et al. 2009). Whether this is true in the case of *Setophaga* warblers and if mate attraction may similarly be disrupted in hybrids is an important component of hybrid zone maintenance that requires further testing.

Conclusion

We demonstrate that opposing dominance effects of ASIP-RALY led to discordant badges of status within hybrid *Setophaga* warblers and ultimately inferior territorial performance. We propose that this finding may indicate an underlying behavioural mechanism contributing to selection which maintains and strengthens the species boundary in this species pair in the early stage of divergence. Additionally, since the pleiotropic effect of the ASIP-RALY gene block on hybrid fitness represents a single genetic region, our results provide valuable insights into the one-locus model of speciation. Such complex behavioral consequences related to a simple genetic process may represent a key mechanism of selection that maintains the species boundary. Future research that tracks genetic processes and social interactions over time would help reveal whether the influence of behavioral dynamics is persistent in the face of gene flow, allowing speciation to proceed.

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