Differences in timing of migration and response to sexual signalling drive asymmetric hybridization across a migratory divide

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Abstract

Ecological traits and sexual signals may both contribute to the process of ecological speciation. Here we investigate the roles of an ecological trait, seasonal migratory behaviour and a sexual trait, song, in restricting or directing gene flow across a migratory divide in the Swainson's thrush (*Catharus ustulatus*). We show that short-distance migratory ecotypes wintering in Central America arrive earlier at the breeding grounds than long-distance migratory ecotypes wintering primarily in South America, providing the potential for some premating isolation. Playback experiments suggest that early- and late-arriving forms recognize each other as competitors, but that the early-arriving form responds more aggressively to a broader spectrum of stimuli. Genetic analysis suggests that hybridization occurs more often between males of the early-arriving ecotype and females of the late-arriving ecotype. Together our results suggest that differences in arrival times may reduce the temporal coincidence of mate choice, but asymmetry in response to heterotypic song may hinder complete divergence. These data provide further insight into the roles of ecological traits and sexual signals during the incipient stages of speciation.

Introduction

There remains a great deal to be learned about the role of ecological traits and sexual signals in the process of ecological speciation (Maan & Seehausen, 2011). In migratory birds, divergence in seasonal migration may affect both pre- and post-mating isolation (Gavrilets, 2003; Price, 2008). It may contribute to premating isolation if individuals mate assortatively based upon arrival time (Bearhop *et al.*, 2005; Rolshausen *et al.*, 2010) and to post-mating reproductive isolation if hybrids with intermediate migratory behaviour are less fit (Rohwer & Manning, 1990; Helbig, 1991; Rohwer & Irwin, 2011). The effects of differences in seasonal migratory behaviour are especially apparent across migratory divides, regions of contact between groups with divergent migratory routes and wintering locations (Berthold *et al.*, 1992; Bensch *et al.*, 1999, 2009; Ruegg,

2008). However, sexual traits such as song are also important to restricting or directing gene flow (Marler, 1960; Searcy & Andersson, 1986; Grant & Grant, 1996; Slabbekoorn & Smith, 2002; Catchpole & Slater, 2003), and divergence in sexual signals can occur as a by-product of ecological adaptation (Ballentine, 2006; Huber *et al.*, 2007; Maan & Seehausen, 2011). Here we investigate the role of seasonal migratory behaviour and habitat-dependent song differences in facilitating or constraining divergence across a migratory divide.

Although temporal isolation is known to be an important reproductive barrier in insects, plants and some fish species (Rundle & Nosil, 2005), there is some uncertainty around the degree to which allochrony represents an important reproductive barrier in migratory birds. Some data suggest that temporal segregation on the breeding grounds can lead to strong assortative mating in migratory birds (Monteiro & Furness, 1998; Bearhop *et al.*, 2005), but other analyses indicate that premating isolation via arrival time may be limited (Rolshausen *et al.*, 2010). Unlike many plant and fish species where fertilization periods are entirely asynchronous (Rundle &

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Nosil, 2005), in birds, arrival times may differ, but breeding periods largely overlap. Thus, even if some individuals mate asssortatively based upon arrival time, temporal isolation may be incomplete in the absence of additional reproductive barriers (e.g. differences in plumage or song, hybrid inviability/infertility) (Rolshausen *et al.*, 2010).

Acoustic signals are also thought to be important during the incipient stages of speciation through their role in species recognition and male–male competition (Marler, 1960; Searcy & Andersson, 1986; Grant & Grant, 1996; Slabbekoorn & Smith, 2002; Catchpole & Slater, 2003; Kirschel *et al.*, 2009). Birdsong can signal male quality and fighting motivation during intraspecific competition for territories and mates (Catchpole & Leisler, 1986; Salomon, 1989; Martin & Martin, 2001; Kort *et al.*, 2008). When males no longer respond to heterotypic song, it can indicate that speciation has already occurred because the two forms no longer recognize each other as competitors (Irwin *et al.*, 2001). Alternatively, an asymmetrical response to heterotypic signals may lead to asymmetrical hybridization if one form is perceived as competitively superior (Pearson, 2000; Pearson & Rohwer, 2000). Consequently, it is critical to understand the functions of song and the impact of acoustic variation on the probability and direction of hybridization between closely related forms across a hybrid zone (Baker & Cunningham, 1985; Slabbekoorn & Smith, 2002; Price, 2008).

Here we investigate the role of arrival times and song in restricting or directing gene flow across a migratory divide in the Swainson's thrush located in the Coastal Mountains of British Columbia (Ruegg & Smith, 2002; Ruegg, 2008) (Fig. 1). On the western side of the divide is *Catharus ustulatus ustulatus,* a short-distance migrant found breeding along the west coast of North America, migrating along a coastal route and wintering in Central America. On the eastern side of the divide is *C. u. swainsoni,* a long-distance migrant found breeding in interior boreal and coniferous forests, migrating along an eastern route and wintering mostly in South America (Evans Mack & Yong, 2000; Ruegg & Smith, 2002) (Fig. 1). Previous results suggest that the two forms co-occur within a narrow tension zone maintained by dispersal and ecologically mediated barriers to gene flow (Ruegg, 2008). The presence of between 27% and 85% parentals in the centre of the hybrid zone (populations 3–6, Table 1; Fig. 1) indicates that the two forms coexist somewhere in the middle of the continuum between a 'good species' and a 'hybrid swarm' (Ruegg, 2008). A species in this intermediate phase of divergence yields a model system in which to investigate the behavioural and ecological traits that may help complete the process of speciation (Nosil *et al.*, 2009).
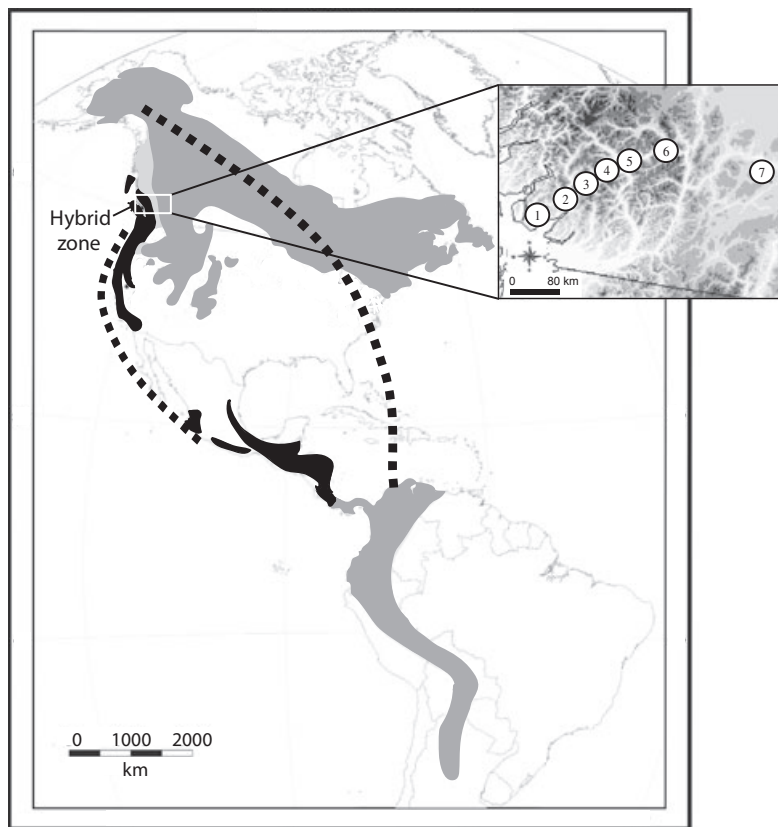

Fig. 1 The Swainson's thrush hybrid zone and migratory divide. The black, light grey and dark grey areas show the hypothesized breeding and wintering areas of *swainsoni* (dark grey), hybrids (light grey), and *ustulatus* (black). The dotted lines represent the hypothesized migratory routes (Ruegg & Smith, 2002). The white box and associated close-up map highlights the location of populations 1–7 across the hybrid zone in British Columbia (population names listed in Table 1).

The association of the Swainson's thrush hybrid zone with an ecological gradient suggests that ecological selection may play a role in restricting or directing gene flow between the two migratory ecotypes. Many recently diverged groups of birds show distinct differences in plumage, suggesting that visual cues may drive premating isolation (Uy *et al.*, 2009a,b). However, in the Swainson's thrush, plumage variation is subtle (*ustulatus* is more russet in colour than *swainsoni*; Ruegg, 2008), but significant differences have been documented in migratory behaviour and song. The short-distance migrating *ustulatus* ecotype is known to arrive approximately 3 weeks earlier than the long-distance migrating *swainsoni* ecotype outside of the hybrid zone (Campbell *et al.*, 1997; Ruegg, 2008). However, arrival dates within the hybrid zone have not been previously assessed. Songs also differ between the groups: the *ustulatus* ecotype from coastal temperate rainforest habitat has longer songs with lower initial frequencies than the *swainsoni* ecotype from drier inland coniferous forests (Ruegg *et al.*, 2006b). Previous research suggests that song differences are driven mainly by habitat differences rather than genetic similarity (Ruegg *et al.*, 2006b). However, it remains untested whether the birds perceive the differences in song and whether the two forms recognize each other as separate species or intraspecific competitors.

Here we gather observational and experimental data to study how arrival times and acoustic signals may impact the extent and direction of gene flow across the Swainson's thrush migratory divide. To assess the potential for temporal segregation in sympatry, we observe arrival times in three populations located at the statistically determined centre of the hybrid zone (Fig. 2) (Ruegg, 2008). To understand the role of song in species recognition and/or male–male competition, we perform playback experiments in *ustulatus* and *swainsoni* populations located in close allopatry (to the east and west of the hybrid zone centre). To assess how divergence in arrival times and song divergence may impact the extent and direction of hybridization, we re-analyse previously published mitochondrial and nuclear DNA data sets from individuals across the hybrid zone.

Materials and methods

Arrival times

To assess the arrival times in sympatry, we monitored the territories at the centre of the hybrid zone (populations 4, 5 and 6; Table 1) during two-two-week intervals – one early in the breeding season, from 10 to 24 May, and one later in the breeding season, from 7 to 21 June. The centre of the Swainson's thrush hybrid zone is characterized by low population density, with ≤ 1 individual per 100-m transect identified during point count surveys of suitable habitat (for details see Ruegg, 2008). Therefore, to have sufficient sample sizes to assess arrival times, we pooled data across the years 2003–2005. From 10 to 24 May, habitat was assessed for new arrivals at a minimum of once every other day by surveying breeding sites in the morning or evening, the two periods when males are most likely to be singing on their territories. Arrival date was defined as the first day that a bird was identified as singing on a territory. Individuals were captured in mist-nets within 1–48 h after being identified on new territories, banded, and genetic samples were taken to confirm subspecies identity or hybrid status using the same nuclear genetic methods described below (see Direction of Hybridization). To the best of our knowledge, every individual present in each of the monitored areas was captured during the first 2-week period, and territories were revisited later in the breeding season to confirm that colour-banded males remained on established territories. To determine the frequency of late-arriving birds, we sampled for new territories from 7 to 21 June, when all birds were present on the breeding grounds. To determine whether there was a significant difference in the frequency of individuals from each category present in the May and June samples, we performed a Fisher's exact test. Recaptures within a season and between seasons were excluded from the analysis so that each observation represents the behaviour of a single individual.

Playback experiments

Playback experiments in which the song of one male is played within the territory of a heterospecific male test the potential for song recognition or competitive dominance between closely related forms (Patten *et al.*, 2004;

Table 1 Assignment of individuals from across the hybrid zone according to maternally inherited mtDNA and bi-parentally inherited nuclear AFLP markers. Individuals with a mismatch between their mtDNA type and their AFLP assignment are evidence of introgression.

		Female-mediated mtDNA	AFLP		
Population	Km from Coast		*ustulatus*	*swainsoni*	Hybrid
1) Sunshine Coast	0	*ustulatus*	29	0	0
		swainsoni	0	0	0
2) Squamish	33	*ustulatus*	6	0	0
		swainsoni	1	0	0
3) Whistler	51	*ustulatus*	8	0	0
		swainsoni	10	0	2
4) Shadow Lake	63	*ustulatus*	9	0	3
		swainsoni	8	1	4
5) Pemberton	70	*ustulatus*	1	0	3
		swainsoni	2	1	8
6) Lillooet	116	*ustulatus*	0	0	0
		swainsoni	0	14	3
7) Kamloops	215	*ustulatus*	0	1	0
		swainsoni	0	18	0

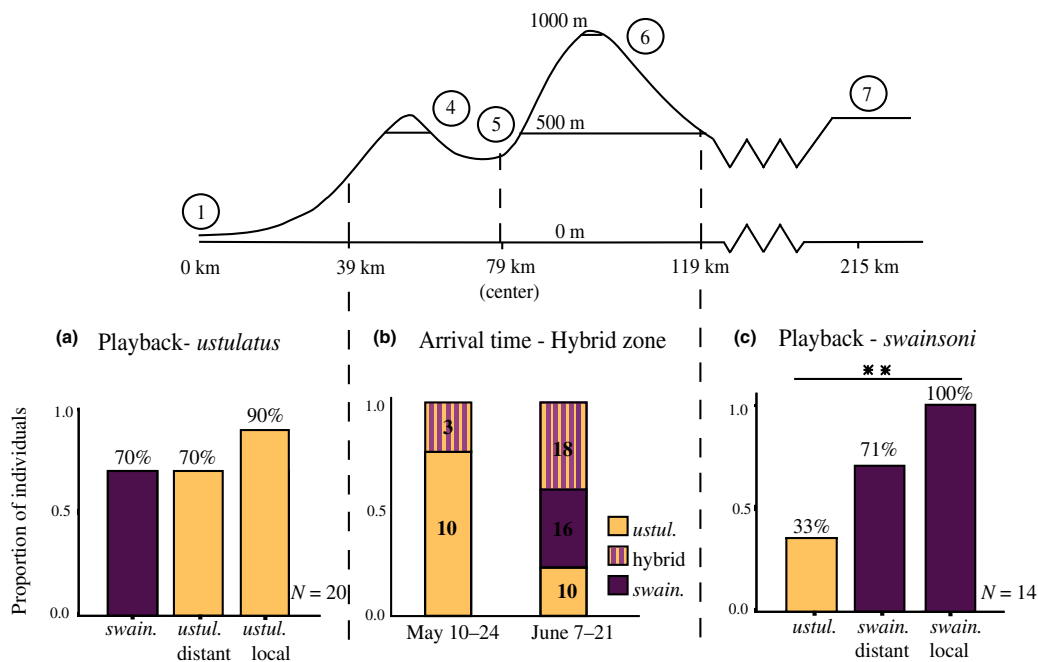AFLP, Amplified Fragment Length Polymorphisms.

Fig. 2 The elevation profile depicts the relative location of populations that were the focus of the playback and arrival time studies (numbers refer to population names listed in Table 1; Fig. 1). Dashed lines indicate the cline centre (79 km from population 1) and width of the cline (from 39 to 119 km), as determined by previous cline analysis (Ruegg, 2008). (a and c) Playback response of *ustulatus* and *swainsoni* males to songs from within and between subspecies. Stars indicate a highly significant *post hoc* comparison following a Wilcoxon signed-ranks test. The results suggest that *swainsoni* males attack significantly more in response to their own subspecies song than to *ustulatus* song, whereas *ustulatus* males attacked regardless of the song type. (b) The proportion and number of individuals from each category present from 10 to 24 May vs. 7 to 21 June. The results suggest that significantly more *ustulatus* individuals are present early in the breeding season.

Dingle *et al.*, 2010). We simulated encounters between males using playback experiments to determine whether males recognized differences in song. We performed an initial set of playbacks to determine the response of males to Swainson's thrush songs from across the breeding range (western to eastern NA) in comparison with songs from the co-occurring, but distantly related warbling vireo, *Vireo gilvus* (see Appendix S1). These experiments demonstrated that attacks, defined as approaches within 1 m of speaker, were reserved for within-species responses. Therefore, the number of attacks was used as the sole response measure in the intraspecific comparisons. Attacks included darting at the speaker, dive-bombing the speaker and sitting on the top of the speaker while actively displaying aggressive wing flutters and other posturing (Dilger, 1956).

Intraspecific playback experiments were conducted on 20 *ustulatus* males from population 1 and 20 *swainsoni* males from population 7 from 28 May to 5 June 2005. In these experiments, males were presented with three stimulus types: local homotypic, distant homotypic and heterotypic song. Each individual was presented with songs from three stimuli categories using a total of 18 stimulus birds, six birds from each stimulus category, by which we optimally avoided the problem of pseudo-

replication (Kroodsma, 1989, 1990). Local homotypic songs were recorded from breeding males within 20 km of populations 1 and 7 (but not near neighbours). Heterotypic songs for *ustulatus* males were recorded from *swainsoni* males in population 7, whereas heterotypic songs for *swainsoni* experiments were recorded from *usutulatus* males in population 1. Distant homotypic songs were recorded from either *ustulatus* or *swainsoni* males from Oregon sites > 500 km from the location of the playback experiments. Recordings were made using a Sennheiser ME67 directional microphone and a Sony TCD-D100 Digital Audio Tape (DAT) recorder with Sony DT-90R tapes.

All playback experiments consisted of two minutes of continuous singing per stimulus type (local homotypic, distant homotypic, heterotypic) separated by 4 min of silence. Digitized recordings were downloaded uncompressed onto an iPod (Macintosh, Inc., Cupertino, CA, USA) and amplified through a SME-AFS Amplified Field Speaker (Saul Mineroff Electronics, Inc., Elmont, NY, USA). The three stimuli were presented consecutively to reduce the variation in response behaviour resulting from differences in weather and time of day. The order that each stimulus type was presented was rotated each time an experiment was preformed on a new bird to

ensure that the order of presentation did not confound the results. Individuals that failed to respond to any stimulus type were removed from the analysis. We performed a nonparametric Friedman test to assess whether there was a significant difference in the frequency of attacks in response to local homotypic, distant homotypic and heterotypic song. Furthermore, we performed *post hoc* Wilcoxon signed-ranks tests with Bonferroni correction for multiple comparisons to identify the variables driving significant 3-way comparisons. Both statistical analyses were performed using the software package SPSS 11 for Mac OSX (SPSS Inc.)

Direction of hybridization

To determine the direction of mating in hybrid individuals at the centre of the hybrid zone, we re-analysed the patterns of maternally inherited mtDNA and bi-parentally inherited nuclear DNA from Ruegg (2008) at the individual level. The details of the Amplified Fragment Length Polymorphisms (AFLP) and mtDNA screening methods can be found in the study by Ruegg (2008). In short, individuals from the centre of the hybrid zone were scored as present or absent for 15 polymorphic AFLP loci and screened for mtDNA clade membership using the PCR-RFLP method described in (Ruegg & Smith, 2002). The software program NEWHYBRIDS, version 1.1 beta 3 (Anderson & Thompson, 2002), was used to assign individuals to pure *ustulatus*, pure *swainsoni* and hybrid categories. Because we could not distinguish between F1, F2 and first-generation backcross categories with our data, these three categories were lumped into one 'early-generation hybrid category'. Individuals that were classified as pure *ustulatus* or *swainsoni* according to their AFLP profile, but had the opposite mtDNA assignment were considered late-generation backcrosses/introgressed. It was assumed that introgression was the result of a hybridization event in the distant past followed by successive backcrossing to one of the parental populations. We performed a chi-square goodness of fit test to determine whether early-generation hybrids and introgressed individuals are more likely to have *ustulatus* or *swainsoni* mtDNA than what would be expected from a pattern of random mating.

Results

Arrival times

Arrival time analyses indicate that short-distance *ustulatus* migrants arrive earlier than long-distance *swainsoni* migrants within the hybrid zone. There was a significant difference in the frequency of *ustulatus, swainsoni* and hybrid birds captured in the early (10–24 May) and late (7 to 21 June) samples ($P < 0.001$) (Fig. 2b). From 10–24 May, across all 3 years, all of the established territories were occupied by either *ustulatus* (77%, $n = 10$) or

hybrids (23%, $n = 3$). However, in the second sample from 7 to 21 June, only 23% of the new territories were occupied by *ustulatus* ($n = 10$), 41% were occupied by hybrids ($n = 18$), and 36% were occupied by *swainsoni* ($n = 16$). Although we do not have enough data to break down our results by year, the fact that in all 3 years (2003–2005) no *swainsoni* individuals were captured during the first 2-week interval indicates that the differences in arrival times are consistent across years. The results were consistent with and without the inclusion of the four hybrids and 14 *swainsoni* individuals from high-elevation population 6 that were all part of the second sample (without population 6, $\chi^2_1 = 6.0$, $P < 0.05$), indicating that *ustulatus* birds arrive earlier than either *swainsoni* or hybrid birds in the centre of the hybrid zone, regardless of the elevation.

Playback experiments

The two main results from the playback experiments are as follows: (i) *ustulatus* and *swainsoni* males respond aggressively to each other's songs, but (ii) there is an asymmetrical pattern of response with *ustulatus* males attacking regardless of the song type, but *swainsoni* males attacking significantly less in response to *ustulatus* than to *swainsoni* songs. A nonparametric Friedman test revealed no significant difference in the response of *ustulatus* males to songs from within and between their subspecies group ($\chi^2_{2,20} = 4.57$, $P = 0.102$) (Fig. 2a), but a significant difference in the response of *swainsoni* males to songs from within and between their subspecies group ($\chi^2_{2,14} = 12$, $P < 0.005$; Fig. 2c). *Post hoc* Wilcoxon signed-ranks tests revealed that the significant 3-way comparison in the *swainsoni* group was driven by the fact that *swainsoni* males attacked significantly more in response to local *swainsoni* song than to *ustulatus* song ($Z = -2.83$, $P < 0.001$) (Fig. 2c), to local *swainsoni* than to distant *swainsoni* song ($Z = -2.00$, $P < 0.05$) and to distant *swainsoni* than to *ustulatus* song ($Z = -2.00$, $P < 0.05$). With Bonferroni corrections for multiple comparisons, the only significant result was a stronger response to local *swainsoni* song than to *ustulatus* song, with the other two-two-way comparisons being reduced to strong trends.

Comparison of nuclear and mitochondrial DNA

Building upon previous analysis (Ruegg, 2008), individuals from across the hybrid zone were separated into three classes (pure, hybrid and introgressed) based upon an analysis of maternally inherited mtDNA and bi-parentally inherited nuclear loci (AFLPs) (Table 1; Fig. 3). Pure individuals had a perfect match between their multilocus nuclear assignment and their mtDNA assignment; hybrids were classified as belonging to F1, F2 or first-generation backcross categories; and introgressed individuals had a mismatch between their multilocus nuclear profile and their mtDNA type.

Here we found that significantly more early-generation hybrids had *swainsoni* mtDNA ($n = 17$) than *ustulatus* mtDNA ($n = 6$) ($\chi_1^2 = 4.5$, $P < 0.05$, Table 1), suggesting that when hybridization does occur, it is asymmetrical. Furthermore, of the 22 introgressed individuals from across the hybrid zone with a mismatch between their mtDNA and multilocus nuclear assignment, 21 of them had *swainsoni* mtDNA on an *ustulatus* nuclear backdrop ($\chi_1^2 = 17.4$; $P < 0.0001$) (Table 1). The results suggest that hybridization between *ustulatus* males and *swainsoni* females predominates over the reciprocal cross and that this mating asymmetry has resulted in introgression of *swainsoni* mtDNA into *ustulatus* populations.

Discussion

There was recently a call for integrative studies of populations at various levels of reproductive isolation to evaluate how ecological traits and sexual signals interact during the process of speciation (Maan & Seehausen, 2011). Here we studied a migratory divide between a long-distance migrant (C. u. swainsoni) and a shorter-distance migrant (C. u. ustulatus) to assess the role of seasonal migration and song in determining the extent and direction of gene flow between closely related forms. We found that *ustulatus*, the shorter-distance, coastal-migrant, arrived earlier at the breeding grounds than *swainsoni*, the long-distance, inland migrant. The response to song playbacks indicated that early-arriving *ustulatus* males and late-arriving *swainsoni* males respond aggressively to each others songs, with *ustulatus* males responding more frequently to a broader spectrum of stimuli. Patterns of maternally inherited mtDNA and bi-parentally inherited nuclear DNA in early-generation hybrids suggest that when reproductive isolation breaks down, it results in hybridization between early-arriving, *ustulatus* males and late-arriving *swainsoni* females.

Temporal segregation in sympatry

Although arrival times in *ustulatus* and *swainsoni* populations outside of the hybrid zone were already known to differ by approximately 3 weeks (Campbell *et al.*, 1997), here we demonstrate for the first time that arrival times also differ in the region where the two forms co-occur (Fig. 2b). One of the main challenges with the arrival time analysis was that low population densities at the centre of the hybrid zone limited the total number of arrival time observations. The evidence to date suggests that the Swainson's thrush hybrid zone is a tension zone in which case low population densities at the centre may relate to the existence of a density trough (Barton & Hewitt, 1985). Additional years of arrival time observations would help increase the sample size and allow for a more complete assessment of how factors such as sex, elevation and climate may impact arrival date. Despite the limited number of observations, the overall pattern of *ustulatus* males arriving first was consistent across years. In addition, our results were consistent with and without the inclusion of the highest-elevation population, demonstrating that elevation differences alone cannot account for the observed pattern.

Previous studies have shown how divergence in migratory and wintering ecology can have a significant impact on breeding ecology in migratory organisms (Marra *et al.* 1998; Bearhop *et al.*, 2005). Here we demonstrate that the timing of arrival on the breeding grounds is connected to the proximity of the wintering location: *ustulatus* birds migrating from Central America arrive earlier than *swainsoni* birds migrating primarily from South America (Ruegg & Smith, 2002). Differences in arrival times on the breeding grounds are potentially important if individuals mate assortatively based upon arrival time, as has been shown in European blackcaps
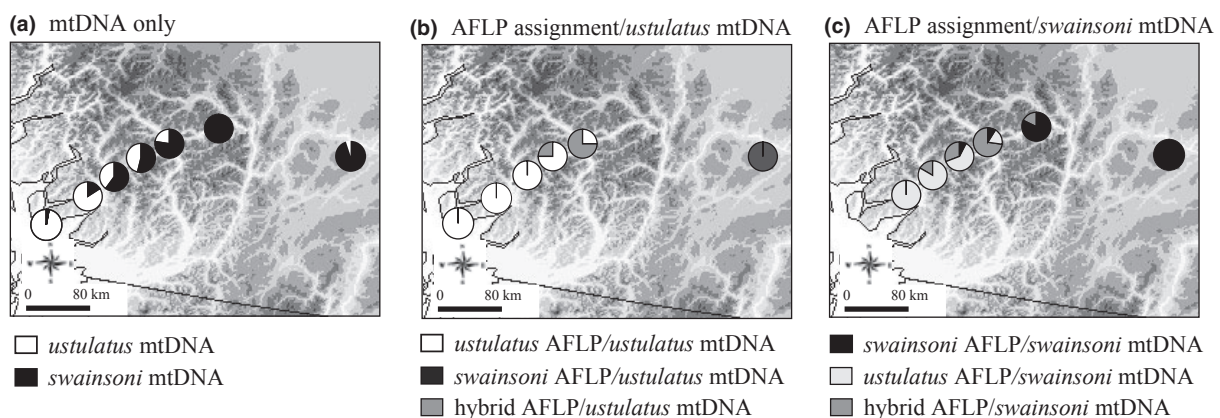

(a) mtDNA only   **(b)** AFLP assignment/*ustulatus* mtDNA   **(c)** AFLP assignment/*swainsoni* mtDNA

☐ *ustulatus* mtDNA
■ *swainsoni* mtDNA

☐ *ustulatus* AFLP/*ustulatus* mtDNA
■ *swainsoni* AFLP/*ustulatus* mtDNA
▨ hybrid AFLP/*ustulatus* mtDNA

■ *swainsoni* AFLP/*swainsoni* mtDNA
☐ *ustulatus* AFLP/*swainsoni* mtDNA
▨ hybrid AFLP/*swainsoni* mtDNA

Fig. 3 Results of the comparison between mtDNA and Amplified Fragment Length Polymorphisms (AFLP) assignment across the hybrid zone showing introgression of *swainsoni* mtDNA in individuals classified as *ustulatus* according to their AFLP profile. (a) mtDNA results modified from Ruegg (2008). (b) AFLP assignment of individuals with *ustulatus* mtDNA. (c) AFLP assignment of individuals with *swainsoni* mtDNA.

(*Sylvia atricapilla*) (Bearhop *et al.*, 2005; Rolshausen *et al.*, 2010). Some premating isolation resulting from differences in arrival times may help explain the presence of a strong barrier to gene flow across the Swainson's thrush hybrid zone (Ruegg, 2008). Our results lend further support to the idea that strong connections between breeding and wintering areas (migratory connectivity) may accelerate the process of divergence in migratory organisms (Berthold *et al.*, 1992; Bearhop *et al.*, 2005; Rolshausen *et al.*, 2009).

Response to sexual signals

The extent to which ecological adaptations and sexual signals promote or hinder further divergence is an area of ongoing research (Maan & Seehausen, 2011). Previous research on the Swainson's thrush system has shown that *ustulatus* and *swainsoni* occupy distinct ecological niches on the breeding grounds, with *ustulatus* occupying temperate coastal rainforest habitat and swainsoni occupying drier, more seasonal inland habitat (Ruegg *et al.*, 2006a). A comparison of genetic, environmental and acoustic distances between the two ecotypes indicates that song differences are driven mainly by environmental variables (Ruegg *et al.*, 2006b). Here we test the extent to which environmentally driven differences in song are perceived by males of both forms. Our results suggest that *ustulatus* males respond equally aggressively to heterotypic and homotypic song types, whereas *swainsoni* males respond more aggressively to homotypic song (Fig. 2a,c).

One hypothesis that may explain the asymmetrical pattern of response to heterotypic song is that *swainsoni* males perceive *ustulatus* males as superior competitors. Previous research has shown that a competitor of similar or lower fighting prowess often evokes the most aggressive behaviour (Thornhill, 1984; Briffa & Elwood, 2001), whereas a competitor of higher fighting prowess will evoke less aggressive behaviour because it intimidates the receiver (Cramer & Price, 2007; Hardouin *et al.*, 2007). Thus, in the case of the Swainson's thrush, asymmetry in aggression may result if *ustulatus* males attack regardless of the song type because they perceive both *ustulatus* and *swainsoni* to be competitors of similar or lesser fighting abilities, whereas *swainsoni* males refrain from attacking in response to *ustulatus* songs because they perceive *ustulatus* males as superior competitors. Competitive superiority of *ustulatus* males could also help explain why our genetic data suggest that *swainsoni* females may hybridize more frequently with *ustulatus* males than the reverse pattern of mating.

A similar pattern of asymmetrical aggression leading to mtDNA introgression has been demonstrated in the Hermit/Townsends warbler hybrid zone (Pearson, 2000; Pearson & Rohwer, 2000, Rohwer 2001). However, in the case of Hermit and Townsend's warblers competitive

dominance has lead to the replacement of Hermit Warblers by Townsend's warblers. In contrast, in the Swainson's thrush all evidence, including historical accounts, suggests that the hybrid zone is a stable tension zone that has settled in a trough of low population density (Ruegg, 2008). One possibility is that in the Swainson's thrush system, *ustulatus* male superiority and asymmetric hybridization have driven the hybrid zone 25 km east of its original centre point (as in Rohwer *et al.* 2001; Krosby & Rohwer 2010), before stalling at a region of low population density. Factors driving hybridization and possible zone movement could be further explored by a comparison between the present British Columbia transect and other possible hybrid zone regions further South identified in Ruegg (2008).

An alternative explanation for the observed asymmetrical pattern of response to song playbacks is that some *swainsoni* males fail to recognize *ustulatus* song. Recognition failure may be exacerbated if *ustulatus* signals, optimized for coastal rainforest habitat, do not transmit well in drier, interior *swainsoni* habitat. If a recognition failure is responsible for the asymmetrical response to song playbacks, then previous research suggests that the strength of the males response should decrease with increasing dissimilarity of the challenger's signal (Nelson, 1988; Dabelsteen & Pedersen, 1992; Colbeck *et al.*, 2010). Although there was a trend towards the strength of *swainsoni* male's response decreasing with increasing dissimilarity of the challenger's signal, this trend was not significant. Further studies in which playbacks are performed on *ustulatus* and *swainsoni* males from similar habitat types at the centre of the hybrid zone would help distinguish between the competitive asymmetry and the recognition failure hypotheses.

Although we cannot clearly distinguish between alternative explanations for differential response to song playbacks, it is clear that both forms respond aggressively to heterotypic song in regions of close allopatry (at the tail end of the hybrid zone). If song recognition is an indication of the extent of divergence, then an aggressive response to playbacks may signal that *swainsoni* and *ustulatus* are in the intermediate stages of speciation where gene flow has been significantly reduced, but they remain competitors for mates and territories. Thus, based on our results we would predict that either a competitive asymmetry or an asymmetry in recognition failure would lead to a breakdown in song as a premating isolation mechanism. Additional playback experiments on males and females from the centre of the hybrid zone would help to further clarify the role of song as a reproductive barrier. It is not known, for example, the extent to which song divergence is exaggerated or reduced at the centre of the hybrid zone (reinforcement-type effects) and/or how females in this zone respond to heterotypic song types.

Asymmetrical hybridization/unidirectional introgression

In the early stages of speciation, reproductive isolation is incomplete, resulting in some hybridization and introgression between closely related forms (Nosil *et al.*, 2009). Our genetic analyses suggest that early-generation hybrids are more likely to have *swainsoni* than *ustulatus* mtDNA. Mitochondrial introgression is a common feature in hybrid zones and can be explained by a variety of mechanisms (reviewed in Wirtz, 1999; Chan & Levin, 2005; Carling & Brumfield, 2009). One explanation is that mtDNA is selectively neutral and therefore flows freely across hybrid zones. However, neutrality would result in a cline widening with the centre position remaining the same (Barton, 1993; Barton & Gale, 1993), and previous research shows that the Swainson's thrush mtDNA cline is similar in width to the nuclear clines, but its centre is shifted 25 km west of the nuclear cline centre (Ruegg, 2008).

Another explanation for mtDNA introgression is that there may be positive selection for *swainsoni* mtDNA west of the hybrid zone centre. A similar scenario has been proposed to explain the movement of arctic charr (*Salvelinus alpinus*) mtDNA into wild brook charr (*S. fontinalis*) populations in eastern Quebec (Canada) (Bernatchez *et al.*, 1995; Glemet *et al.*, 1998; Doiron *et al.*, 2002). In the charr system, it is hypothesized that Arctic charr mtDNA may provide a metabolic advantage to high-altitude populations of wild brook charr. However, in the Swainson's thrush system it is unclear why higher altitude *swainsoni* mtDNA would provide a metabolic advantage in the lower altitude temperate coastal range of *ustulatus*.

Although we cannot rule out the possibility of selection on the mtDNA, our data on arrival times and the response to song playbacks lead to the prediction that *ustulatus* may have an advantage in territory and mate selection that could drive asymmetrical hybridization. In migratory organisms, early-arriving males have first access to the best breeding sites (Hasselquist, 1998) and often have higher reproductive success (Moller, 1994; Lozano *et al.*, 1996). In our study, we did not observe any turnover in territory occupancy with the arrival of later *swainsoni* males and it is possible that late-arriving *swainsoni* males were forced to occupy lower quality territories (although a thorough assessment of this was beyond the scope of the present work). If *ustulatus* males receive advantages for early arrival, the occasional preference of *ustulatus* males by late-arriving *swainsoni* females may help explain the limited and biased direction of gene flow across the migratory divide. Furthermore, if differences in the response to song playbacks are the result of a competitive asymmetry, then females may prefer competitively dominant males. Additional research documenting the territory quality, patterns of female choice and mating success within the hybrid zone would further elucidate the factors driving mtDNA introgression.

Conclusions

A thorough understanding of the incipient stages of speciation requires the study of multiple reproductive barriers (Rundle & Nosil, 2005; Lemmon & Lemmon, 2010; Qvarnstrom *et al.*, 2010). Although migratory behaviour and song may play an important role in restricting and directing gene flow between the two migratory ecotypes, there remain many unexplored reproductive barriers that require further investigation. These include the potential influence of previously documented differences in body size and plumage coloration on prezygotic isolation (Ruegg, 2008) as well as the influence of post-zygotic isolating mechanisms such as hybrid inviability/sterility and Haldane's Rule (reviewed within Rundle & Nosil, 2005). Thus, it is possible that migratory behaviour and song may play a disproportionately large role in premating isolation, but additional analysis is needed to test this idea. Of particular interest within the context of a migratory divide is the potential influence of intermediate migratory behaviour on the fitness of hybrid offspring (Rohwer & Manning, 1990; Helbig, 1991; Rohwer & Irwin, 2011). Investigating each of these alternative reproductive barriers provides fertile area for future research.

If ecological speciation is viewed as a multistep process (Nosil *et al.*, 2009), then *ustulatus* and *swainsoni* may be considered migratory ecotypes in the intermediate stages of speciation where ecological divergence has occurred, but reproductive isolation is incomplete. Whether migratory ecotypes will continue to diverge into separate species depends upon the nature of the divergent selection. Selection on the migratory programme is thought to be especially powerful because it can lead to exploitation of novel ecological niches (Berthold *et al.*, 1992), thus driving selection at multiple traits important to ecological speciation (Rolshausen *et al.*, 2009). However, it is yet to be determined whether the interaction between ecological traits and sexual signals inhibits or promotes speciation (Maan & Seehausen, 2011). Here we show that although differences in arrival times may reduce the temporal coincidence of mate choice, an asymmetry in the response to heterotypic song may hinder complete divergence. These data provide further insight into the potentially multifaceted behavioural mechanisms underlying ecological speciation.

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Supporting information

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Appendix S1 Interspecific playback methods.

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