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Isotopic variation across the Audubon's–myrtle warbler hybrid zone

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Abstract

Differences in seasonal migratory behaviours are thought to be an important component of reproductive isolation in many organisms. Stable isotopes have been used with success in estimating the location and qualities of disjunct breeding and wintering areas. However, few studies have used isotopic data to estimate the movements of hybrid offspring in species that form hybrid zones. Here, we use stable hydrogen to estimate the wintering locations and migratory patterns of two common and widespread migratory birds, Audubon's (*Setophaga auduboni*) and myrtle (*S. coronata*) warblers, as well as their hybrids. These two species form a narrow hybrid zone with extensive interbreeding in the Rocky Mountains of British Columbia and Alberta, Canada, which has been studied for over four decades. Isotopes in feathers grown on the wintering grounds or early on migration reveal three important patterns: (1) Audubon's and myrtle warblers from allopatric breeding populations winter in isotopically different environments, consistent with band recovery data and suggesting that there is a narrow migratory transition between the two species, (2) most hybrids appear to overwinter in the south-eastern USA, similar to where myrtle warblers are known to winter, and (3) some hybrid individuals, particularly those along the western edge of the hybrid zone, show Audubon's-like isotopic patterns. These data suggest there is a migratory divide between these two species, but that it is not directly coincident with the centre of the hybrid zone in the breeding range. We interpret these findings and discuss them within the context of previous research on hybrid zones, speciation and migratory divides.

Introduction

Selection against hybrid offspring between divergent taxa can be a potent form of reproductive isolation (Barton & Hewitt, 1985), as demonstrated theoretically and in laboratory experiments (Coyne & Orr, 2004). While in some systems the ecological factors that influence hybrid fitness are known (e.g. Vamosi & Schluter, 1999), studying the fate of hybrids in wild populations is much more challenging. This is particularly true of migratory birds, a group in which closely related species often differ dramatically in migratory behaviour and wintering ranges (Irwin & Irwin, 2005; Price, 2008). For instance, hybrids may be ill suited to ecological conditions on their breeding grounds, on their migratory routes or in their overwintering areas, and these areas may occur over large geographic scales (Delmore *et al.*, 2012). Identifying the relative importance of these different areas to hybrid fitness, while challenging, has important implications for our understanding of hybridization in the wild (Price, 2008).

While new tracking technology such as light-level geolocators has improved our ability to follow migratory birds over their annual movements (reviewed in McKinnon *et al.*, 2013), there are a number of constraints that make their use impractical in some study systems, including the limited capacity of many small songbirds to carry such a device. Alternatively, stable isotopes have been used with some success in other

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avian systems, particularly in Europe, to indirectly estimate the overwintering locations of offspring formed in hybrid zones (Bearhop *et al.*, 2005; Inger & Bearhop, 2008). For instance, in *Phylloscopus* willow warblers in Sweden, stable nitrogen and carbon in feathers (Chamberlain *et al.*, 2000) has been combined with band recovery (Bensch *et al.*, 1999), orientation studies (Ilieva *et al.*, 2012) and genetic data to illustrate an excellent example of a 'migratory divide' (Bensch *et al.*, 1999, 2009). In this case, these data suggest that the two subspecies migrate to, and winter in, different areas of Africa. Birds in a contact zone between these subspecies are, on average, intermediate in their isotopic signature, although it is not clear whether this represents a mixture of the parental overwintering locations or a distinct, and potentially intermediate, area in which the hybrids overwinter (Chamberlain *et al.*, 2000; Bensch *et al.*, 2009). In a different European species complex, hybrids between pied and collared *Ficedula* flycatchers illustrate a different pattern: carbon and nitrogen isotopic data suggest that most of the hybrids winter in a location similar to only one of the parental species (Veen *et al.*, 2007), although new isotopic data suggest this relationship may be more complex than originally described (Veen *et al.* in press).

There is much evidence that the migratory programme of small songbirds is inherited genetically (Berthold, 1996; Helbig, 1996; Pulido, 2007; Irwin, 2009). Therefore, in the case of willow warblers, mixed or intermediate migratory behaviours may be inherited and expressed in hybrids. Such behaviours may be inferior, taking individuals into inhospitable areas, suggesting that this may be an important component of postmating reproductive isolation in the willow warbler contact zone (Chamberlain *et al.*, 2000; Bensch *et al.*, 2009). Alternately, in the case of the flycatchers, the authors attribute the clustering of hybrids with one of the parental species to a dominant inheritance pattern of migratory behaviour and suggest that such a pattern might explain the high overwinter survival of hybrids (Veen *et al.*, 2007). In both cases, estimating these behaviours in the zone of secondary contact using isotopic data proved important for understanding the evolutionary dynamics in the hybrid zones.

Isotopic patterns across avian hybrid zones have not previously been studied in North America, which is in fact very well suited for such studies because of the extensive knowledge of the geographic variation of feather isotopes, especially stable hydrogen (Hobson 1999; Meehan *et al.*, 2004; Bowen *et al.*, 2005; Hobson *et al.*, 2012). Here, we present isotopic data across a narrow and stable hybrid zone between Audubon's (*Setophaga auduboni*) and myrtle (*S. coronata*) warblers in western Canada (Hubbard, 1969; Barrowclough, 1980; Brelsford & Irwin, 2009; note that the American Ornithological Union currently treats these as two subspecies within the broad species 'yellow-rumped warbler'; the International Ornithologists' Union instead identifies the two as distinct species; here we use the latter treatment). These two migratory songbirds are distinguished by their breeding ranges and distinct plumage patterns (Fig. 1a): myrtle warblers breed mostly east of the Rockies and have a white throat and a black cheek patch; Audubon's warblers breed mostly west of the Rockies and have a yellow throat and a grey face (Hunt & Flaspohler, 1998). Where the ranges of the two overlap, in the Rocky Mountains between British Columbia and Alberta, they form a narrow hybrid zone (Hubbard, 1969; Barrowclough, 1980; Brelsford & Irwin, 2009). This is also in an area where a number of other avian

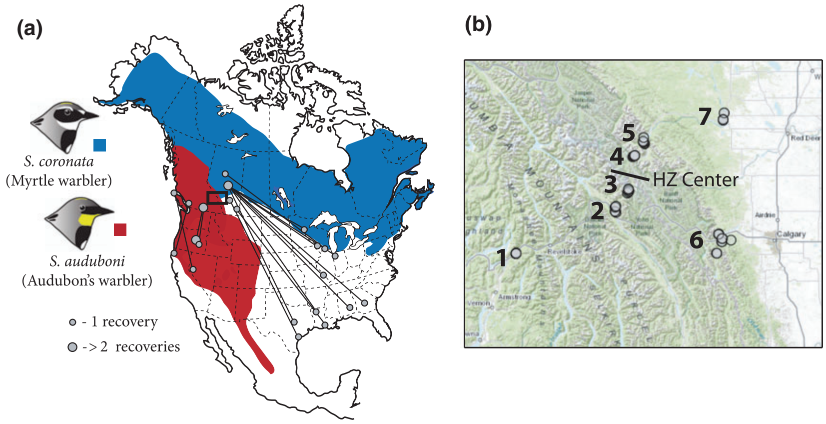


Fig. 1 (a) Distribution and band recoveries of Audubon's and myrtle warblers. Banding data obtained from Brewer *et al.* (2006) and the Canadian Bird Banding Office (2013). Note that there is a distinct subspecies of Audubon's warbler, the black-fronted warbler, that occurs in Mexico that is not distinguished on this map. (b) Sampling localities across the Audubon's/myrtle warbler hybrid zone. Site 1 is defined here as an allopatric Audubon's population; site 7 is an allopatric myrtle population; sites 2–6 are within 70 km of the hybrid zone centre. Hybrid zone sites are along the Saskatchewan River Crossing transect (panel D in Fig. 1 of Brelsford & Irwin, 2009), except site 6, which is along the Kananaskis transect (panel E in Fig. 1 of Brelsford & Irwin, 2009).

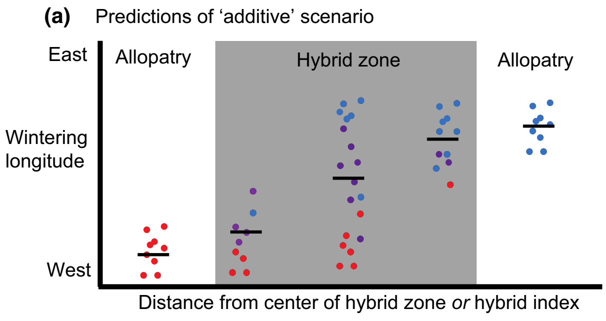
contact zones and hybrid zones are located (Toews & Irwin, 2008; Irwin *et al.*, 2009; Toews *et al.*, 2011). In the Audubon's/myrtle warbler hybrid zone, hybrids and backcrosses are formed at high frequency and individuals with a wide variety of intermediate plumage patterns are common (Hubbard, 1969; Brelsford & Irwin, 2009). The narrowness of the hybrid zone, estimated to be 132 km wide, combined with the estimated root-mean-squared dispersal distance of these species (~20 km), suggests that some form of selection against hybrids maintains the hybrid zone, which Brelsford & Irwin (2009) estimate to be moderately strong, with hybrids having 18% lower fitness than parental forms.

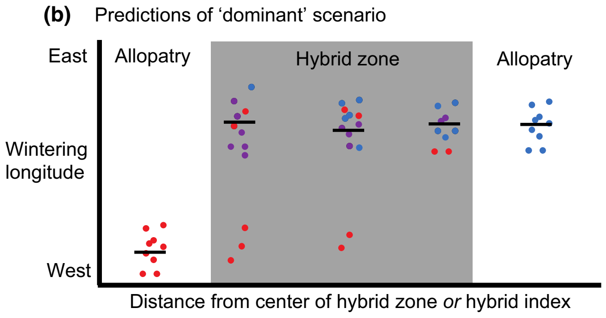
Studies of mate pairing, based on plumage and genotype correlations between social mates, indicate that there is little or no assortative mating in this hybrid zone (Brelsford & Irwin, 2009). Preliminary studies of song and playback responses (Brelsford, 2010) suggest that there are not strong differences between the species in vocalizations. There is also no obvious ecological transition that is coincident with the whole length of the hybrid zone, suggesting that habitat selection is unlikely to be a strong reproductive barrier. Taken together, these findings imply that none of these traits are likely to serve as sizeable premating reproductive barriers between the two species. Hence, post-mating selection on hybrids may be an important form of reproductive isolation maintaining this hybrid zone, but this has been studied in much less detail. For instance, we have no direct data on developmental success, behaviour or annual survival of hybrids.

An important challenge to understanding hybrid fitness in this system is that it is unclear where hybrids spend the winter months and how they move between their breeding and wintering ranges. Field observations illustrate that Audubon's warblers winter along the Pacific coast from southern British Columbia, Canada, through to Baja California, highland Mexico and Central America (see overwintering densities from citizen science surveys in Fig. S1). Myrtle warblers winter in the south-eastern USA, concentrating around Louisiana, with wintering populations also occurring along the Pacific coast, along the gulf coast in Central America and in the Caribbean (Hunt & Flaspohler, 1998). Limited mark/recapture data involving sites near the hybrid zone are consistent with these observations (Fig. 1a): recovery data suggest that myrtle warblers banded or encountered near Calgary and Edmonton, Alberta, tend to move to and from the south-eastern USA, some moving as far as Texas, Louisiana or Georgia to overwinter (Fig. 1a; Brewer *et al.*, 2006). In contrast, Audubon's warblers banded or encountered in south-central B.C. and along the Pacific coast travel south through Oregon and some into California. Field data for hybrids are scarce, primarily because hybrids can be difficult to identify and distinguish from the parental species in their basic plumage in the fall and winter. In addition, at present most citizen science surveys and bird-banding stations do not identify hybrids.

Here, we analyse variation in stable hydrogen (δ^2H) across the hybrid zone between these two species to determine whether there is a change in migratory behaviour across the hybrid zone and to gain a better understanding of where hybrids are spending the winter. To our knowledge, this is the first study to present isotopic variation on such a fine spatial scale in a narrow hybrid zone. We use hydrogen because it forms a strong gradient over much of North America, which varies with patterns of precipitation and is primarily influenced by distance to the ocean and elevation (Bowen *et al.*, 2005). This results in particular values of hydrogen in certain areas, including the areas thought to be wintering habitats for myrtle and Audubon's warblers. In particular, areas in the south-eastern USA have, on average, higher hydrogen values (i.e. more deuterium) as compared to areas along the Pacific coast (Hobson *et al.*, 2012). More importantly, hydrogen has also been the subject of previous investigations linking environmental hydrogen to hydrogen in feathers of known origin, allowing us to broadly estimate the geographic region where a feather was grown (Hobson *et al.*, 2012).

We first tested whether allopatric Audubon's and myrtle warblers winter in isotopically distinct areas, as is implied by field observations and banding recovery data. We were interested in testing whether there was evidence of a migratory divide between these two species, whether it was coincident with the centre of hybrid zone and whether any transition in overwintering behaviour was narrow or gradual. We then studied variation amongst hybrids in the hybrid zone in more detail. There are a number of possible patterns expected from hybrids, depending on their overwintering location and the distribution of hydrogen in these areas. We have simplified these predictions into three nonmutually exclusive scenarios (Fig. 2). The first, which we call the 'additive' scenario (Fig. 2a), has been observed in hand-raised blackcap warblers (*Sylvia atricapilla*; Helbig, 1991). These studies found a high variance in hybrid blackcap migratory behaviour and an overall intermediate directionality of their migratory movements. If hybrid Audubon's and myrtle warblers exhibited such a pattern, we would expect that hybrids might migrate to and overwinter in areas similar to both parental species as well as areas intermediate between the two. An intermediate route or wintering area between the main parental overwintering concentrations (i.e. coastal California/Baja and the south-eastern USA) is predicted to be near southern Arizona and New Mexico, western Texas and northern Mexico. While intermediate in geography between the two main overwintering concentrations, these areas in fact have lower isotopic values than is observed in either of the parental taxa, at least based on environmental


(a) Predictions of ‘additive’ scenario


(b) Predictions of ‘dominant’ scenario

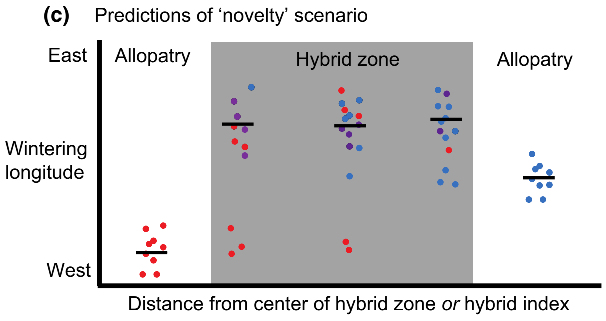

(c) Predictions of ‘novelty’ scenario

Fig. 2 A simplified schematic of the general predictions for the transition in overwintering behaviour across a hybrid zone. Each point represents an individual’s estimated wintering longitude with its distance relative to the centre of the hybrid zone and coloured by its genetic background. (a) shows the predictions consistent with an ‘additive’ scenario, where hybrids show a high variance, an overall intermediate behaviour and a correlation between their genetic background and overwintering location. (b) illustrates the ‘dominance’ pattern, where individuals overlap in their overwintering location with one or the other of the parental species. (c) shows a pattern where hybrids overwinter in an entirely novel areas as compared to either of the parental species.

isotopic variation across North America identified previously (Bowen *et al.*, 2005). Under this scenario, we would also expect a correlation between a bird’s migratory behaviour and its genetic background. Under the ‘dominant’ scenario (Fig. 2b), which has been suggested for flycatchers (Veen *et al.*, 2007) and reed warblers (Yohannes *et al.*, 2011), hybrids winter in areas that overlap with one of the two parental species. Finally, in the ‘novelty’ scenario (Fig. 2c), hybrids winter in an entirely unique area, which may or may not be intermediate between the two parental species. In these two latter scenarios, the relationship between genetic background and migratory behaviour is rather complex in comparison with the additive scenario. More generally, our goal here is to estimate any differences in overwintering location between these two parental species and then test how these behaviours might be expressed in hybrids between them. Such studies are important, given that most avian hybrid zone research focuses on individuals during the breeding season.

Materials and methods

Sampling and genotyping

During the spring of 2006 and 2007, we captured 70 myrtle warblers, Audubon’s warblers and hybrids in the hybrid zone and in allopatric populations in eastern British Columbia and western Alberta. We studied territorial individuals along a previously studied transect near Saskatchewan River Crossing, Alberta (sites 1–5 and 7 in Fig. 1b; this is the same transect as illustrated in panel D of Fig. 1 in Brelsford & Irwin, 2009), and from each site we sampled 6–16 individuals (average $n = 10$ birds). We also included samples from a site near the centre of the hybrid zone but through a different transect, near Kananaskis, Alberta (site 6 in Fig. 1B; panel E in Fig. 1 in Brelsford & Irwin, 2009). We did this to provide a comparison of variation between transects, and this was also the site of a series of orientation experiments in 2011 (see Discussion). While sampling additional allopatric populations would be ideal, to address our central questions our sampling emphasized studying individuals within the hybrid zone. For each population, we included the relative distance from the centre of the hybrid zone, which was estimated from a previous genetic and plumage study (Brelsford & Irwin, 2009). These hybrid zone parameters are consistent across space and time (Hubbard, 1969; Brelsford & Irwin, 2009).

We captured individuals using song playback and mist nets and then took photographs, feather samples and a blood sample (10–40 μL) from each individual. Birds were captured following territory establishment and most prior to nesting at a time when they are most responsive to song playback. To each bird we applied a unique numbered metal leg band before releasing the individual. All animal care was conducted according to the University of British Columbia protocols. We aged and sexed all individuals according to Pyle (1997); most captured birds were males, due to the use of song playback to attract and capture them, but 5 of the 70 individuals were female.

Blood samples, taken using a small needle and capillary tube from the brachial vein, were stored in Queen’s lysis buffer (Seutin *et al.*, 1991) and left at ambient temperature until returned to the laboratory for analysis of genotypes. DNA was extracted using a phenol–chloroform protocol and resuspended with

50–200 μL of buffer (depending on the size of the pellet) containing 10 mM Tris–HCl and 1 mM EDTA, at pH 8.0, and stored at 4 °C. Genotype information for two of the three nuclear markers (*CHD1Z* and *numt-Dco1*) was presented in a previous publication (Brelsford & Irwin, 2009), where the full PCR and genotyping protocol can be found. For the third nuclear marker, *RIOK2*, we used the forward primer ATGGGTGTTGGCAAAG AATC and the reverse primer GCTCCTCTTCRTTWGCA ACA and used a PCR annealing temperature of 60 °C. This amplifies an 850-base pair fragment, which is cut by the enzyme *XmnI* in Audubon's warblers. Previous research suggests that all three markers are at or near fixation for alternate alleles in the two species, with marker frequencies < 0.1 for the myrtle variant in Audubon's warblers and vice versa for CHD1Z, < 0.1 for *numt-Dco1* and < 0.2 for *RIOK2* (Brelsford & Irwin, 2009). Note that *RIOK2* and *CHD1Z* are both Z-linked markers and thus only have a single allele in females. Such linkage can also produce nonindependence, especially if there is little recombination between the markers. Given that only 45% of individuals previously studied along this transect have the same allele types (i.e. Audubon's vs. myrtle) for the two markers, there is still much added information with including both Z-linked markers, although excluding *RIOK2* or *CHD1Z* does not alter the conclusions of the study. To generate a genetic hybrid index, we added each myrtle allele for the three markers and divided this by the total number of alleles (6 for males and 4 for females), resulting in an index that ranges between 0 (all Audubon's alleles) and 1 (all myrtle alleles).

Feather isotope analysis

For each bird, we determined the stable hydrogen content (δ^2H) in one greater covert feather, pulled from the inner side of the prealternate moult limit. Stable hydrogen here refers to the relative amounts of the two stable forms of hydrogen (deuterium over protium) divided by that ratio in a standard material:

$$\partial^2\mathrm{H} = \left[\frac{\frac{^2\mathrm{H}}{^1\mathrm{H}}x}{\frac{^2\mathrm{H}}{^1\mathrm{H}}S} - 1\right] \times 1000$$

where x is the isotope ratio of the sample and S is the isotope ratio of the standard. Feathers were analysed at Environment Canada's Isotope Laboratory at the National Water Research Institute in Saskatoon, SK. We took advantage of the prealternate feather moulting pattern in yellow-rumped warblers, where at the end of the winter these birds moult many of their body feathers and 3–4 of their inner-covert feathers on their wintering grounds during a prealternate moult. Therefore, a bird captured on its breeding territory will have freshly moulted feathers from the prealternate moult that contain the isotopic signature of its most recent wintering area or an early potion of its migration route (see Discussion; Gaddis, 2011).

Statistical analyses

To test whether feathers from the two allopatric populations differ in their stable isotope composition, we used a two-sample *t*-test in R (R Core Team, 2014), as distributions appeared roughly normal with similar variances. To study the distribution of isotopic variation amongst hybrid genotypes, we used a linear discriminant function analysis to group the individuals based on the isotopic variation in the allopatric sites. We first used the DFA to test the classification of each of the allopatric birds to determine the general rate of misclassification. We then used this function to classify hybrid individuals as either Audubon's-like or myrtle-like based on their isotopic values. This procedure produces posterior probabilities of assignment to each of the groups, with individuals close to the overlap between Audubon's and myrtle isotopic values having similar probabilities of being assigned to either group. To test for a correlation between hybrid index and isotopic value, we used the binomial classifications from the DFA, which assigned individuals to Audubon's or myrtle isotopic groups, in a generalized linear model. This was done only for those individuals within the hybrid zone, excluding those from the allopatric populations and from the Kananaskis site. We performed a logistic regression using the 'logit' link function in R to test whether the posterior assignment output (response variable) of the DFA was associated with genetic hybrid index (explanatory variable).

We estimated the geographic origin of the feathers using IsoMAP, a framework that allows for modelling, predicting and analysis of stable hydrogen isoscapes (Bowen *et al.*, 2014; http://www.isomap.org). The system draws on hydrogen analysed from environmental water collected at stations distributed globally for over 30 years to model fine-scale geographic variation in isotopes. We first created a geostatistical precipitation model of environmental hydrogen (the dependent variable) as a function of CRU-derived precipitation data (Mitchell & Jones, 2005) and ETOPO elevation data (independent variables; U. S. National Geophysical Data Center, 1998) over the years 1980–2008 (IsoMAP JobKey: 31437). We included environmental isotopic data collected from April to October as previous studies show that growing season correlates more strongly with hydrogen values than other time periods (Bowen *et al.*, 2005; Hobson *et al.*, 2012). This model was then used to estimate spatial variation of hydrogen across the USA and Central America within a longitudinal range of 131.5° to 61°W and a latitudinal range of 3.3° to 51.5°N (IsoMAP jobkey: 31439).

In most cases, there is not a 1 : 1 relationship between hydrogen in precipitation ($\delta^2\mathrm{H}_\mathrm{p}$) and organic samples, such as feather keratin (δ^2H_f), because of how hydrogen moves through food webs, and it is therefore important to generate an empirically based transfer function between the two (Bowen *et al.*, 2014). This is done using known-origin samples, feathers in this case, to estimate environmental isotope equivalents, which can then be used to estimate similar environmental isotopic values from feathers of unknown origin (Bowen *et al.*, 2014). To do this, we used data from 209 feather samples previously analysed by Hobson *et al.* (2012) where the location of feather growth was known. Our subset of the Hobson *et al.* (2012) data included only migratory species, of which the majority (> 75%) were from species in the Parulidae family (i.e. wood warblers). We then used QGIS (Quantum GIS Development Team, 2013) to estimate the environmental equivalent for each of the known-origin samples from the environmental hydrogen surface created by IsoMAP (Fig. S1). IsoMAP assumes a linear relationship between the feather and environmental water isotope function, and we used the equation $\delta^2H_f = 1.3450 \times \delta^2H_p - 20.17$ (see additional details in deriving this function in the Supplementary materials). Following Bowen *et al.* (2014), this can be algebraically rearranged to estimate the environmental hydrogen value from feather isotopes of unknown origin.

From this relationship, we estimated the environmental hydrogen equivalents in each of our feather samples. Using IsoMAP, we then generated a geographic likelihood assignment surface for each individual. We used the 'individual assignment' function and included each individual's environmental-equivalent hydrogen value plus the standard deviation of the residuals from the water/feather transformation function (9.96‰). The resulting likelihood surfaces were then standardized for each site. We did this by finding the highest maximum likelihood amongst individuals within a site and then linearly scaling each individual's likelihood surface to have this same maximum likelihood in QGIS (Quantum GIS Development Team, 2013). The standardized surfaces were then combined, additively, for each individual within a site. We did this standardization because one component of an individual's likelihood surface includes the absolute number of pixels on the landscape that have similar isotopic values. Therefore, if there are only a few pixels on the map with a bird's isotopic value, it is assigned a high likelihood, whereas if there are a large number of pixels with a similar isotopic value, it divides the likelihood amongst those pixels. In some cases, this results in variance in the maximum likelihood amongst individuals within a site and, if not standardized, biases any combination of the surfaces to those individuals having isotopically unique values within the spatial extent of the isotope map. Standardizing the individual likelihoods to have the same maximum likelihood means that individuals are treated similarly in this regard, resulting in a better illustration of the geographic patterns considering all individuals at a site. Therefore, the relative distribution of the resulting likelihood values for any given standardized map is informative, although the absolute values are not an accurate representation of any individual's assignment uncertainty. These standardized likelihood surfaces for each site were illustrated with QGIS (Quantum GIS Development Team, 2013). To illustrate areas of potential geographic overlap between the assignment of the pure myrtle and Audubon's sites, we also calculated the absolute difference between these two standardized likelihood surfaces.

Results

Samples from populations outside the hybrid zone (sites 1 and 7 in Fig. 1) differ significantly in their mean isotopic signature ($t_{15} = 4.01$, $P = 0.001$). This was in the expected direction of the allopatric myrtle population having a significantly higher mean δ^2H (mean $\delta^2H = -42.6$‰) compared with the Audubon's warbler population (mean $\delta^2H = -63.6$‰). Isotopic variation amongst individuals and sites within the hybrid zone is more complex, and variation in the hybrid sites generally overlaps with that of allopatric populations, with the most overlap with myrtle warblers (Fig. 3). Sites 3–5 have a similar mean and distribution as compared to allopatric myrtle warblers (site 7), whereas site 2 has a more intermediate mean and high variation, with a mixture of isotopic values observed in the pure Audubon's and myrtle warbler sites in addition to values well outside the range of both (e.g. −113.6‰ and −9.2‰). Individuals sampled from site 6 (the diamonds in Fig. 3), along the Kananaskis transect, have overall higher hydrogen values (average −29.5‰) compared with the parental species as well as with individuals from other hybrid zone sites.

Isotopic variation within genetic classes is consistent with the geographic patterns: most of the hybrids have distributions of isotopic signatures that are similar to that of pure myrtle warblers (Fig. 3). The discriminant function analysis (Fig. 4) has an overall accuracy of assignment for the allopatric birds of 82%. Due to the high hydrogen values, all of the individuals sampled from the Kananaskis transect were assigned to the myrtle group. From the other hybrid zone sites (i.e. sites 2–5), 76% of the individuals were assigned to the myrtle-like isotopic group (28 of 37 birds), with an average δ^2H of −39.9‰ (SD = 10.4). The rest were assigned to the Audubon's-like isotopic group (nine of 37 birds), with an average isotopic value of −68.8‰ (SD = 18.1). Within the hybrid zone (sites 2–5), there was no significant relationship between genotype and isotopic assignment ($z_{35} = -1.55$, $P = 0.12$).

The likelihood assignment estimating the growing regions of feathers based on δ^2H differs between the

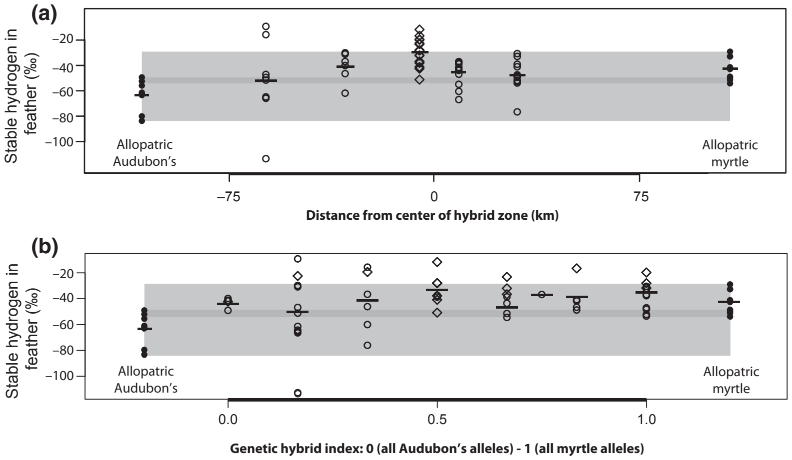


Fig. 3 Stable hydrogen and genetic variation across the Audubon's/myrtle warbler hybrid zone. The filled symbols indicate the two allopatric populations (Audubon's and myrtle, left and right, respectively); the open circles indicate individuals from sites 2–5 along the Saskatchewan River Crossing transect, and the diamonds indicate individuals sampled from site 6 along the Kananaskis transect. The black lines represent the mean for each group, and the grey boxes represent the range of variation observed in the two allopatric populations; the darker portion represents the overlap between these two distributions. In (a) the isotopic value of each individual is shown relative to its distance from the centre of the hybrid zone (in km). Brelsford & Irwin (2009) estimate the zone to be 132 km wide. In (b) the points are grouped by an individual's genetic hybrid index, derived from three nuclear markers (*CHD1Z*, *RIOK2* and *numt*). There was no significant relationship between an individual's genotype and its isotopic assignment for birds in the hybrid sites ($z_{35} = -1.55$, $P = 0.12$).

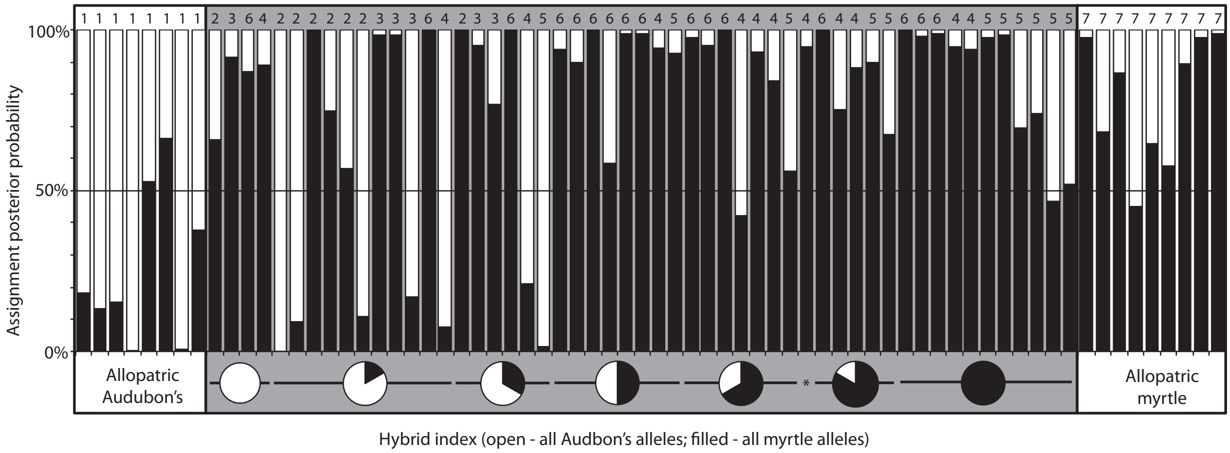


Fig. 4 Posterior probability of individuals assigned as either Audubon's-like (white) or myrtle-like (black) based on isotopic data from a discriminant function analysis. Each column represents a single individual with the proportion of white or black representing the probability of the DFA assigning its isotopic value as Audubon's-like or myrtle-like, respectively. Individuals in the hybrid zone are distinguished by the hybrid index, a combination of three nuclear genetic markers. The numbers above each column represent the site that an individual was sampled from (see Fig. 1).

pure Audubon's and myrtle feathers (Fig. 4). Feathers from myrtle warblers are assigned to areas that are restricted primarily to the south-eastern USA and the Caribbean (Fig. 5c). In contrast, the likelihood surface for Audubon's is highest in other regions, although showing altogether more uncertainty with regard to areas of geographic assignment. In this case, birds have high likelihood of wintering in regions including coastal California and the Pacific Northwest, the south-west, as well as a long band that crosses the Great Plains and extends to the Atlantic coast (Fig. 5b). There are two primary regions where we identified quantitative similarities in the assignment probabilities between these two allopatric groups: a large region in the north (extending from the southern Rocky Mountains north into Canada) and a narrow band extending from the Atlantic, along the southern USA and into Central America (Fig. 5d). The areas in the north and

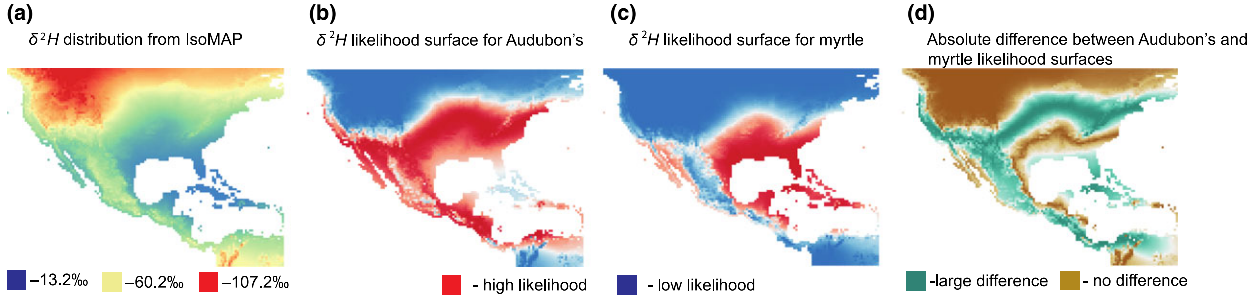


Fig. 5 (a) Stable hydrogen prediction isoscape generated from IsoMAP (Bowen *et al.*, 2014). (b) and (c) are the likelihood assignment surfaces for allopatric Audubon's and myrtle warblers, respectively. (d) The absolute difference in the standardized likelihood surfaces between myrtle and Audubon's warblers. Brown areas illustrate areas of isotopic overlap; green areas show areas where the likelihood surfaces differ between the two.

north-west are areas where both allopatric groups have extremely low (i.e. near zero) assignment likelihoods. The more southerly, narrow band is instead an area where both groups have similarly low, but nonzero, assignments.

Feathers from individuals in the hybrid zone, sampled from sites 3–5 (Fig. 6b–d), have the highest likelihood assignment to areas in the south-eastern USA. This area extends west to Texas, north to Illinois, east to the Atlantic and south to the Gulf Coast of Central America and into the Caribbean. A smaller proportion of individuals also have areas of assignment further west, including Baja and north-western Mexico. Feathers from site 6 (Fig. 6e), sampled from birds in the Kananaskis transect and showing particularly high hydrogen values, have assignments that are shifted even further into the south-east, with the most likely areas of assignment, including southern Florida and Cuba. The likelihood surfaces for site 2 (Fig. 6a) suggest a much more diffuse pattern, with geographic assignments for these birds that include the south-east, the south-west and along the Pacific coast.

Discussion

Here, we present the first study of isotopic variation in the Audubon's/myrtle warbler hybrid zone. The location of this zone, along the Rocky Mountains of eastern British Columbia and south-western Alberta, Canada, is in an area where a number of other avian contact zones and hybrid zones are located, and migratory behaviour has been postulated to play a role in maintaining some of them (Toews & Irwin, 2008; Irwin *et al.*, 2009; Toews *et al.*, 2011; Rohwer & Irwin, 2011). Stable hydrogen data from birds captured on their breeding territories, but in feathers grown at or near their most recent wintering grounds, provide the first estimates of the overwintering location of hybrid offspring between these two species. The data suggest that Audubon's and myrtle warblers, at least in populations near the hybrid zone studied here, winter in isotopically distinct areas (Figs 3 and 5). We also found a number of novel and complex patterns in the isotopic data from hybrids. Most hybrids appear to be wintering in the south-eastern USA, overlapping with the known wintering areas for myrtle warblers (Fig. 6b–d), although others are also likely wintering in the west (i.e. some individuals from site 2; Fig. 6a) and some even further to the south-east (i.e. individuals from site 6; Fig. 6e). Most of the isotopic variation related to where an individual bred in the hybrid zone, and there was no detectable correlation with a bird's genetic background. We interpret these findings below and discuss them within the context of previous research on hybrid zones, speciation and migratory divides.

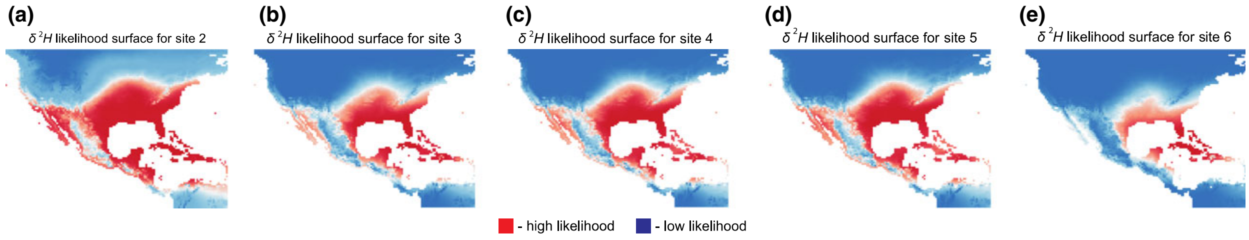


Fig. 6 (a) to (e) Likelihood assignment surfaces for individuals sampled in the hybrid zone, from sites 2 to 6. Given these are standardized likelihood surfaces (i.e. each individual has a surface that is scaled to have the same maximum likelihood), the relative likelihood distributions are informative, but the absolute values have little meaning (see Methods).

The result that allopatric Audubon's and myrtle warblers on either side of the hybrid zone differ significantly in the stable hydrogen composition of their feathers suggests that there is a transition in overwintering location between these two species and that it is quite narrow. The likelihood surfaces estimating the geographic origin of the feathers grown during the prealternate moult of birds in the allopatric populations generally agree with previous data from band recoveries: feathers from Audubon's warblers have a high likelihood of originating in coastal California, Baja and south-western Arizona (Fig. 5b) and myrtle feathers likely originated in the south-eastern USA (Fig. 5c). However, the δ^2H likelihood assignment areas are larger than field observations and band recovery data suggest, at least for Audubon's warblers. For instance, field data suggest that Audubon's warblers are rarely observed east of Colorado, New Mexico or Texas during winter months (Hunt & Flaspohler, 1998; Stephenson & Whittle, 2013). This band of high likelihood for Audubon's feathers originating in the central and eastern USA is likely a function of the distribution of δ^2H over the landscape (Fig. 5a) as opposed to being reflective of their true overwintering location. This is also likely true with the area of high likelihood of Audubon's feathers originating in the southern parts of Central America. While there are records of Audubon's wintering in these areas (Hunt & Flaspohler, 1998), it seems unlikely that individuals from our sample overwintered in these localities, although it cannot be excluded. Future studies sampling individuals captured on the wintering grounds will be important to address such uncertainty (e.g. Bensch *et al.*, 2006).

It is important to note that while consistent with field observations, the wintering sites of myrtle (and potentially hybrid) birds in our study may be more southerly than is implied by the feather isotopic data. This is because myrtle warblers may undergo their prealternate moults during the early stages of migration as opposed to directly in the overwintering area (Gaddis, 2011). Data from a banding station in Oregon suggest that approximately 95% of Audubon's warblers had already gone through their prealternate moult as they showed up on spring migration (Gaddis, 2011). In contrast, 60% of myrtle warblers had gone through their prealternate moult when captured on migration, although it is not known whether these birds in Oregon are also representative of birds further to the east or hybrids (Gaddis, 2011). We suspect that myrtle warblers that moult on the wintering grounds (and not on migration) would likely have a higher hydrogen value. This is primarily because hydrogen decreases with latitude in the eastern USA (Fig. 5a). Therefore, if a myrtle warbler began its prealternate moult during the early parts of spring migration, north of its wintering area, it would be expected to have a lower hydrogen value and would appear more 'Audubon's-like' in our analysis. While this would not alter the general conclusions of our study, such a pattern could be confirmed by a more detailed study of moult and isotopic patterns of individuals on spring migration in the eastern USA. One method to address this would be to genotype warblers killed from building strikes on migration. Birds that strike windows on migration make up a large fraction of many tissue collections in museums; therefore, genetic samples are readily available in many cases. This would allow for an objective estimation of the regions used on migration by myrtle and Audubon's warblers.

The data from birds within the hybrid zone suggest that most hybrids likely overwinter in the south-eastern USA (Fig. 6). This can be observed in both the spatial patterns of isotopic variation in the hybrid zone and amongst the various hybrid genotypes (Fig. 2). These patterns can be explained in two ways. First, the individuals from these sites could be growing their feathers in a similar area to myrtle warblers, as we suggest. Alternatively, given the broad regions of high likelihood assignment, hybrids and pure forms could in fact winter in distinct parts of this area. While unlikely, we cannot exclude these possibilities, and such uncertainty may be resolved with additional isotopic data (i.e. carbon, nitrogen and/or oxygen).

There are at least two evolutionary mechanisms that could produce a pattern of most hybrids clustering with one or the other of the parental species. First, dominant inheritance of migratory traits could produce a pattern of most of the hybrids having myrtle-like traits if Audubon's migratory loci were recessive. Such patterns of migratory dominance have been suggested previously in hybrid pied and collared flycatchers in Europe (Veen *et al.*, 2007) and hybrids between great reed warblers (*Acrocephalus arundinaceus*) and clamorous reed warblers (*A. stentoreus*), although in the latter case the number of hybrids sampled was limited (Yohannes *et al.*, 2011). Alternately, the loci responsible for the behaviours may be discordant with the transition in other traits, potentially due to introgression. It has been shown previously that mtDNA has likely introgressed from myrtle into Audubon's warblers at some point in the past (Milá *et al.*, 2011; Toews *et al.*, 2014), and there is some evidence of nuclear introgression between the species (Brelsford *et al.*, 2011). Whether this clustering pattern is due to dominance or discordance of the responsible loci is not clear, but such a result implies that few loci would be involved, given that genetic and phenotypic data do not suggest a general pattern of dominance or introgression of myrtle genes or traits (Brelsford & Irwin 2009; Brelsford *et al.*, 2011). Having few responsible loci seems counterintuitive based on the complexities of a multifaceted trait such as migration, but is not without precedence (Helbig, 1996; Sutherland, 1998), and future genomic studies will be able to address this more conclusively in the future (Leidvogel *et al.*, 2011). We acknowledge, however, that each of these explanations assumes that the migratory direction and overwintering location are under genetic control, as previous studies would suggest (Alerstam, 2006). However, a more ecological explanation is also possible, where individuals respond to features on the landscape, such as the orientation of the Rocky Mountains, which cue them to move in various directions. In this case, we would expect to observe greater variation in the stable isotopic values within a site, which was instead quite consistent across most of the study sites (i.e. average standard deviation of δ^2H of $-11‰$ across all sites, excluding site 2). More generally, we suggest there would clearly be a benefit in performing controlled laboratory studies to estimate the extent of genetic control of migration in this or other migratory species in North America, although this would clearly be a logistical challenge.

Amongst the hybrid zone sites, the clustering of hybrid individuals with the allopatric myrtle site is consistent from sites 3 to 5. In contrast, the individuals sampled at site 6, along the Kananaskis transect (the diamonds in Fig. 3), while overlapping with the other hybrid zone sites, have on average higher hydrogen values compared with samples taken from the main Saskatchewan River Crossing transect. This may be because these individuals, sampled at breeding populations further south in Alberta, also go further southeast, maybe as far as the Caribbean, for the winter (Fig. 6e). This preliminary observation could be confirmed with additional analyses of samples taken from other transects across the hybrid zone. Another notable pattern is at site 2, approximately 60 km west from the hybrid zone centre, which shows much greater isotopic variation than the other sites (Figs 3a and 6a). Our sampling here is too limited to test for a bimodal distribution of values, but the values here overlap with what is observed in both allopatric sites as well as some values that are outside the parental ranges. The finding of higher variance in this potential transition area is consistent with previous studies showing much greater variation in hybrid blackcap migratory orientation behaviours compared with the parental species (Helbig, 1991). Future sampling along the western side of the hybrid zone could provide a more precise estimate of where the shift in isotopic signatures occurs. Indeed, such an observation raises interesting questions about the dynamics of a potential migratory transition on the edge of the hybrid zone.

Across all of the individuals in the hybrid zone, we found no evidence of a strong correlation between the hybrid index and isotopic values (Fig. 4). Two recent studies in blackcap warblers (Rolshausen *et al.*, 2013; Mettler *et al.*, 2013) also found either nonsignificant or weak correlations between genetic markers and isotopic data. It should be noted, however, that our results depend in large part on the accuracy of the hybrid index. Similar results were obtained using the plumage index as opposed to the genetic hybrid index (i.e. most hybrid classes cluster isotopically with myrtle warblers; results not shown). Such plumage traits are likely influenced by numerous genetic loci, although the inheritance patterns of these traits are not known. While the three nuclear markers presented here are sufficient to distinguish hybrids and backcrosses from pure individuals, future studies employing genome sequencing (i.e. Davey *et al.*, 2011) would clearly be much better at identifying additional hybrid classes. This method might also have the resolution to potentially identify some of the important genomic regions linked to these divergent migratory patterns (Leidvogel *et al.*, 2011).

These results suggest a combination of our original predictions, where most hybrid sites show a clustering with one of the parental species (the 'dominance' prediction; sites 3–5), while one shows potentially a novel isotopic patterns (the 'novelty' prediction; site 6) and another shows a high variance and mixture of both parental values (the 'additive' prediction; site 2). Taken together, these data suggest that there is likely a transition in migratory behaviour across the Audubon's/myrtle hybrid zone, but that it is not coincident with the centre of the hybrid zone and is instead shifted to the western edge. Migratory divides, where changes in migratory behaviour are coincident with other characteristics, are most notable in willow warblers (Bensch *et al.*, 2009) in Sweden and in Swainson's thrushes in western Canada (Ruegg, 2008). Migration has been suggested as a potent form of reproductive isolation in these divides: naïve young hybrids may inherit directional cues (Helbig, 1991) that cause them to migrate to an intermediate and potentially inferior wintering location or take them over inhospitable areas during migration (Chamberlain *et al.*, 2000). While new technology has expanded the range of patterns expected from such divides (i.e. loop migration; Delmore *et al.*, 2012), our data from the Audubon's/myrtle hybrid zone suggest that an intermediate migration route and wintering location of most hybrids are not likely.

While an intermediate route or overwintering site is unlikely for most hybrids, these data do not rule out the possibility that migration and overwintering site selection could still impose some fitness consequences for hybrids. For instance, overwintering in habitat suitable for parental species may still impose a challenge to hybrid individuals, which have a mixture of both Audubon's and myrtle genes. It is also important to note that if there is strong selection against hybrids in their first year, it would not be identified by this type of analysis, as the current sample is of those individuals that have successfully migrated south-and-back again. Indeed, it may be that some individuals are heading to an intermediate overwintering location and are encountering less suitable habitat as compared to those further west or east and not returning. Densities of birds in these intermediate and inland areas are lower than those along the coast further east and west (Fig. S1), consistent with the suggestion that these habitats are of potentially lower quality. For example, in southern Arizona, Terrill and Ohmart (1984) found that Audubon's warblers were facultative residents during the winter months: in winters with favourable food availability, more birds remained to overwinter at particular sites. In winters with reduced food availability, however, birds facultatively continued their autumnal migration south to Mexico. This suggests that the winters associated with these inland and higher elevation sites may be more variable than those along the coasts and, consequently, these areas may be less suitable.

In addition to an intermediate overwintering location, employing an intermediate migratory route could potentially take hybrid Audubon's and myrtle warblers over inhospitable areas. For instance, an intermediate route might take hybrid warblers over the middle and southern Rocky Mountains or arid regions, such as the deserts of the south-western USA. This has been proposed as an important ecological barrier that hybrid Swainson's thrush may encounter and, as a consequence, has been proposed as an important post-mating reproductive isolation in this and other systems (e.g. Rohwer & Irwin, 2011; Delmore *et al.*, 2012). There could also be an interaction with moult timing, where individuals have the migratory behaviour of one species but the timing or moult pattern of the other (Rohwer & Irwin, 2011). Finally, hybrids from site 6 heading further into the south-east, potentially into the Caribbean, may experience more extreme weather events during the winter months that could be a potent form of selection. While much of this is speculation, clearly additional technology would be ideal to address more detailed questions such as these. Given that current methods rely on recapturing an individual in subsequent years, inferring the fate of hybrids will necessarily be indirect, until GPS-like tags are small enough to affix to small songbirds.

In conclusion, these isotopic data suggest that myrtle and Audubon's warblers in populations near the hybrid zone winter in distinct locations. They also suggest that most hybrids between the species overwinter in an area that generally overlaps isotopically with myrtle warblers. While there appears to be a transition between the two migratory behaviours, it is shifted to the west of the hybrid zone centre. We suggest this pattern is not supportive of a classic migratory divide, where a shift in migration is coincident with transitions in other phenotypic traits and genetic markers (e.g. Delmore *et al.*, 2012) and where there is a greater potential for selection against hybrids employing an intermediate migratory route. These patterns should be confirmed by additional studies in this and other species groups. In particular, such studies would be useful in determining how common the various patterns of overwintering location (i.e. additive, novel or dominant) are amongst different species and how variable such behaviours are within species. In addition, it is important for future studies to ask how possible differences in overwintering location influence migratory behaviour in the spring and fall, possibly by using orientation studies of birds on route. By integrating patterns of genetics, behaviour and trace elements, our goal is to gain a better understanding of the role of such complex phenotypes in the process of local adaptation and reproductive isolation. They also highlight the importance, and the challenge, of considering an organism's full annual cycle when studying such hybrid zones.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Frequency of all checklists within a given grid cell that identified Audubon's (a) or myrtle (b) warblers between the months of December – February from 2004–2014.

Figure S2 Regression of known-origin feather samples from Hobson *et al.* (2012) with environmental hydrogen.

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