High individual repeatability and population differentiation in stable isotope ratios in winter-grown collared flycatcher *Ficedula albicollis* feathers

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For migrants, we often lack complete information of their spatial distribution year round. Here, we used stable carbon, nitrogen and hydrogen isotope ratios extracted from feathers grown at the wintering sites of the long-distance migratory collared flycatcher *Ficedula albicollis*, to study how individuals from different breeding populations are distributed at the wintering sites. A sub-sample of birds was also sampled in two consecutive years to test for the repeatability of isotope ratios. Birds from the same breeding populations had more similar isotope ratios compared to birds from other nearby populations (10–100 km apart). Furthermore, isotope repeatability within individuals was high, implying that the observed pattern of isotope variation is consistent between years. We put forward two hypotheses for these patterns; 1) strong wintering site philopatry and migratory connectivity, suggesting that migratory connectivity may potentially be found on a much smaller spatial scale than previously considered, and 2) consistent interpopulation differentiation of feeding ecology at their wintering site.

Different phases in a migrant’s annual cycle are co-dependent (Fretwell 1972), and it is generally assumed that carry-over effects between seasons may be wide spread, having important consequences for the ecology, evolution and conservation of migratory organisms (Gill et al. 2001, Webster et al. 2002, Norris 2005). Despite the presumed importance of the wintering phase in a migrant’s life cycle for species studied extensively during the breeding season, we often lack detailed knowledge on where they spend the winter and how they are distributed on the wintering grounds.

The spatial distribution of animals is a key factor in many ecological and evolutionary processes as it, for example, determines with whom individuals interact. The degree of isolation between migrating populations is referred to as migratory connectivity (Webster et al. 2002). Total mixing of breeding populations at the wintering site, as suggested for North American black-throated blue warblers *Dendroica caerulescens*, (Rubenstein et al. 2002) and completely separated wintering sites, as shown in the aquatic warbler *Acrocephalus paludicola*, breeding in Europe (Pain et al. 2004) represent the extremes of a weak and strong migratory connectivity, respectively.

So far, studies of migratory connectivity generally focused on relatively large-scale patterns, involving populations breeding several thousands of kilometres apart (Webster et al. 2002). It is important to note that migratory connectivity is in fact a scale dependent measure: when studied at an increasingly coarser scale, migratory connectivity will appear to become stronger. Conversely, we would expect strong migratory connectivity at a small geographic scale to have increasing biological relevance. For example, strong migratory connectivity could influence the dynamics of meta-populations by reducing gene flow between nearby populations. On the other hand, weak migratory connectivity may facilitate gene flow between populations wintering at different places while breeding in sympatric populations. For example, immigration from Swedish populations of barn swallows *Hirundo rustica*, to breeding grounds in Denmark have been suggested to result in swallows breeding in sympatric populations while having separate wintering grounds (Møller and Hobson 2004). Further, one key component to migratory connectivity is philopatry (Webster et al. 2002). Strong migratory connectivity requires that individuals are faithful to both their breeding and wintering area. However, individuals from populations with weak migratory connectivity could still be philopatric to a specific wintering-site, despite mixing of populations.

Knowledge concerning the spatial distribution of migrants at both breeding and wintering sites is thus crucial for our understanding of a migrant’s life-history. However,
studying migratory organisms at different places throughout the annual cycle is hampered by the difficulty of locating both the wintering and breeding ground of individual migrants. Stable isotopes is one among a series of new techniques for avian ecology studies (Inger and Bearhop 2008), that has been successfully used to e.g. verify differences in wintering sites within (e.g. Bearhop et al. 2005) and between populations (Pain et al. 2004), subspecies (Chamberlain et al. 2000) and hybridizing species and their offspring (Veen et al. 2007). Furthermore, it has been used to document differences in winter habitat quality (e.g. Marra et al. 1998). In this study, we compared stable isotopes of carbon, nitrogen and hydrogen in winter-grown feathers of the long-distance migratory collared flycatcher *Ficedula albicollis*, collected from individuals in five nearby breeding populations on the island of Gotland in the Baltic Sea (maximum distance of 100 km between populations). This allowed us to test how migrants are distributed on their wintering sites in relation to their distributions on the breeding grounds. Additionally, we estimated the repeatability of isotopic patterns by comparing isotopic signatures of the same individuals collected in two consecutive years.

**Methods**

**Study species and data collection**

The collared flycatcher is an insectivorous long-distance migratory passerine spending the winter in sub-Sahara Africa as confirmed by ringing recoveries, visual observations (Cramp 1992) and carbon and nitrogen isotope signatures (Veen et al. 2007). Collared flycatchers have a complete moult after the breeding season and a partial moult, including all tertial feathers during the winter (Cramp 1992, Salewski et al. 2004). The median tertial feather from collared flycatchers breeding on the island of Gotland (57° 10’N, 18° 20’E; 3,140 km²), Sweden, was collected for carbon, nitrogen and hydrogen stable-isotope analyses and the isotope ratios in these feathers were therefore assumed to reflect local environmental conditions at the wintering grounds during the time of moult. Samples (one feather from each bird) were taken towards the end of the breeding season, a few days prior to pre-migratory moult when these feathers are moulted to avoid potential negative effects. Feathers from 89 individuals, 43 males and 46 females, were collected in 2004 (45) and 2005 (44) from five different breeding populations (Fig. 1). In addition, we could identify and sample 21 of the 89 individuals in both 2004 and 2005 (11 males and 10 females). Breeding data (first-egg laying date, clutch size (i.e. number of eggs per nest) and number of fledged juveniles per nest) from these populations were also collected. Birds were caught following the guidelines of the Swedish Bird Ringing Centre (permit number 644). The Swedish National Board for Laboratory Animals approved the collection of feathers (permit number M 78–05).

**Stable isotope analysis**

*Carbon and nitrogen*

The ratios of the stable carbon (¹³C and ¹²C), and nitrogen (¹⁵N and ¹⁴N) isotopes in feather samples (washed in chloroform) were analysed at the Centre for Limnology of the Netherlands Institute of Ecology. Carbon stable isotope ratios (parts per thousand, ‰ difference from the ¹³C/¹²C ratio in Vienna PeeDee limestone; henceforth referred to as δ¹³C) and nitrogen stable isotope ratios (‰ difference from the ¹⁵N/¹⁴N ratio in atmospheric N₂; henceforth referred to as δ¹⁵N) were determined in a HEKAtech EuroEA elemental analyzer coupled on-line through a Finnigan con-fl o interface to a Finnigan Delta S isotope ratio mass spectrometer. Samples were analyzed in a fully randomized order. Average reproducibility based on replicate measurements was <0.2 ‰ for both δC and δN.

*Hydrogen*

Stable hydrogen isotope measurements were analysed on a Thermo Finnigan Delta XP mass spectrometer equipped with a TC-EA pyrolysis furnace in the Faculty of Earth and Life Sciences at the Vrije Universiteit Amsterdam, The Netherlands. Feather samples and keratin standards (~0.1–0.15 mg) were placed into silver capsules and introduced into the TC-EA reactor at 1,450°C resulting in quantitative conversion to H₂ gas, which is separated from other pyrolysis products in a GC column and subsequently analysed in the mass spectrometer. Deuterium values are...
A MANOVA was conducted with data analysis only had nine repeated measures across years. Average reproducibility based on replicate measurements of these standards was $< 4\%$. Samples were again analyzed in a fully randomized order. Note that we only were able to analyze $\delta D$ from 33 out of the 89 individuals and therefore only had nine repeated measures across years.

**Data analysis**

A MANOVA was conducted with $\delta^{13}C$, $\delta^{15}N$ and $\delta D$ as the response variables to test if individual differed in signatures compared to birds from other breeding populations. Additionally, the full model included: 1) sampling year as a fixed factor to control for year effects, 2) sex as a fixed factor to test for any potential sex-specific segregation, and 3) first-egg laying date, body weight (0.1 g) and body size (tarsus length (0.1 mm)) as covariates to test if individual quality affects spatial distribution at the wintering site. An ANCOVA for each element ($\delta^{13}C$, $\delta^{15}N$ and $\delta D$) with the other elements as covariates were subsequently conducted to assess the relative contribution of the response variables while controlling for correlations between them (Quinn and Keough 2002). Given the relatively small sample size for $\delta D$ we also ran a MANOVA followed by two ANCOVAs, as described above, excluding $\delta D$ and thereby using all available data for $\delta^{13}C$ and $\delta^{15}N$.

To select the final model in all above analyses, backward exclusion approaches were used where non-significant effects were consecutively dropped from the models starting with the least significant effects. Each dropped effect was then added back to the final model, one at a time, to validate the final models and confirm that they had no significant impact on them.

We also analyzed the repeatability of $\delta^{13}C$, $\delta^{15}N$ and $\delta D$ between subsequent years. This was done by calculating the Pearson's correlation coefficients (which in this case is analogous to the intra-class correlation coefficient) between isotope ratios from feathers collected from the same 21 individuals in both 2004 and 2005 (only nine were analysed for $\delta D$). To test for potential year-effects, a two-tailed paired $t$-test was conducted for each element separately. Finally, and also for each element separately, we tested if male and female collared flycatchers differed in wintering-site philopatry. To this end, the absolute differences in isotope ratios between years were calculated, followed by two-tailed $t$-tests comparing males and females.

Two males were polygynous and for these cases one of the breeding attempts was randomly assigned to these males. The software package JMP 5.0 was used for all statistical analyses.

**Results**

Isotope ratios were more similar within than among breeding populations (MANOVA: Wilks Lambda, $F = 3.73$, $P = 0.002$). None of the other explanatory variables (see methods) significantly added in explaining the observed variation (all $P > 0.35$) and were therefore dropped from the final model. In the second step we assessed the relative contribution of each of the three response variables from the MANOVA. Differences between breeding populations were found for $\delta^{15}N$ and $\delta D$ (Fig. 2) and there was a tendency for differences between the sexes in their $\delta^{15}N$ ratios (Table 1). All other effects were non-significant and dropped from the final models (ANCOVA: all $P > 0.28$). Furthermore, neither $\delta^{15}N$, $\delta D$ or other effects significantly explained any of the variation in $\delta^{13}C$ (ANCOVA: all $P > 0.5$). The MANOVA and two ANCOVAs using all available data for $\delta^{13}C$ and $\delta^{15}N$ showed similar and significant differences between populations explained by between population differences in $\delta^{15}N$ (results not shown). The near significant effect of sex on $\delta^{15}N$ variation was now significant (ANCOVA: $F = 6.48$, $P = 0.013$) with the increased sample size.

The repeatabilities of $\delta^{13}C$ ($r = 0.76$, $P < 0.001$, $n = 21$) and $\delta^{15}N$ ($r = 0.80$, $P < 0.001$, $n = 21$) were high (Fig. 3), implying that the patterns found above for $\delta^{13}C$ and $\delta^{15}N$ are consistent between years. No tendency for any repeatability of $\delta D$ was found ($r = 0.00$, $P = 0.97$, $n = 9$). $\delta^{13}C$ differed significantly between years (two-tailed paired $t$-test: mean difference ($\pm SE$) = 0.33%$_{oo}$ (0.13%), $t_{20} = 2.5$, $P = 0.021$) while there was a near-significant tendency for a year effect for $\delta^{15}N$ (two-tailed paired $t$-test: mean difference ($\pm SE$) = $-0.44\%_{oo}$ (0.23%$_{oo}$), $t_{20} = -1.9$, $P = 0.072$). Differences for $\delta D$ between years were small and non-significant (two-tailed paired $t$-test: mean difference ($\pm SE$) = $-0.24\%_{oo}$ (5.13%$_{oo}$), $t_{20} = -0.95$, $P = 0.37$).

![Figure 2. Nitrogen ($\%_{oo}$ $\delta^{15}N$) and hydrogen ($\%_{oo}$ $\delta D$) stable isotope ratios in winter-grown median tertial feathers of individual collared flycatchers from five breeding populations (circles are population mean isotopic signature $\pm SE$ on Gotland (numbers next to each circle refer to the label for each breeding population, see Fig. 1). Individuals from the same breeding population had more similar “winter”-isotope ratios compared to birds from other populations. The observed difference between populations was explained by differences in $\delta^{15}N$ and $\delta D$ but not by $\delta^{13}C$.](image)
Table 1. To assess the relative contribution of each response variable from the MANOVA three ANCOVAS were conducted, one for each element, with the other two elements as covariates. Below are the F and P values from the final models presented (all dropped effects had \( P > 0.28 \)). \( \delta^{13}\mathrm{C} \) is not presented as no variable significantly explained variation in \( \delta^{13}\mathrm{C} \) (all \( P > 0.35 \)). Pop stands for breeding population.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \delta^{15}\mathrm{N} )</td>
<td>6.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Sex</td>
<td>2.5</td>
<td>0.120</td>
</tr>
<tr>
<td>( \delta^{2} \mathrm{D} )</td>
<td>8.4</td>
<td>0.008</td>
</tr>
<tr>
<td>Pop.</td>
<td>4.7</td>
<td>0.004</td>
</tr>
<tr>
<td>( \delta^{15}\mathrm{N} )</td>
<td>10.0</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**Discussion**

We found that individuals acquire very similar carbon and nitrogen isotope ratios in their feathers over consecutive years. Furthermore nitrogen and hydrogen isotope ratios were similar for individuals from the same population. How should these patterns be interpreted? In terrestrial habitats, variation in \( \delta^{13}\mathrm{C} \) is mainly attributed to the ratio of primary producers using either C3 or C4 photosynthetic pathways although variation within primary producers in water efficiency also effects \( \delta^{13}\mathrm{C} \) variation (reviewed by Kelly 2000). The lack of differentiation in \( \delta^{13}\mathrm{C} \) between breeding populations could suggest that their wintering areas are situated in roughly the same general area. \( \delta^{15}\mathrm{N} \) variation is more difficult to interpret as many explanatory factors may act simultaneously (reviewed by Kelly 2000). Terrestrial plants may vary widely in their \( \delta^{15}\mathrm{N} \) (e.g. Shearer et al. 1983) mainly due to soil differences (e.g. Shearer and Kohl 1989). There is also a tendency for \( \delta^{15}\mathrm{N} \) in plants to be higher in arid regions (e.g. Shearer et al. 1983), which has consequences for \( \delta^{15}\mathrm{N} \) at higher trophic levels. In addition, as shown in mammals, higher \( \delta^{15}\mathrm{N} \) can result from water stress (Cormie and Schwarze 1996), potentially increasing the association between \( \delta^{15}\mathrm{N} \) and habitat aridity (Kelly 2000, but see Kempster et al. 2007).

Food webs may have their own specific \( \delta^{2} \mathrm{D} \) where differences in \( \delta^{2} \mathrm{D} \) often reflect variations in deuterium levels in the water bodies on which these food webs rely (Lajtha and Michener 1994). Large-scale meteorological processes may create spatial gradients of \( \delta^{2} \mathrm{D} \) (Bowen et al. 2005). The local and temporal variation in \( \delta^{2} \mathrm{D} \) across the collared flycatcher’s moultng range in Africa (Lundberg and Alatalo 1992) also appears to be large, similar to what was suggested for African wintering barn swallows (Møller and Hobson 2004). Using IAEA’s WISER database (accessible via http://nds121.iaea.org/wiser we calculated a \( \delta^{2} \mathrm{D} \) as large as 30 \( \% \) across all available monthly mean February and March \( \delta^{2} \mathrm{D} \) data in precipitation across the collared flycatcher’s moultng range. Also Langin and colleagues (2007) concluded that during a single year, at a single study site 60 ha small in North America, variation \( \delta^{2} \mathrm{D} \) in juvenile American redstart feathers was equivalent to a geographical variation of up to 900 km. It has therefore been argued that \( \delta^{2} \mathrm{D} \) should be used only to assign individual birds to broad geographic moultng zones (Langin et al. 2007). The differentiation between populations in \( \delta^{2} \mathrm{D} \) in this study seems to be caused by one population deviating from the others, potentially suggesting that collared flycatchers from this population may winter away from the other populations. However, inferring what spatial scale these isotopic differences between populations are related to should be done with caution and be considered speculative. The differences between populations observed in this study are based on differences in both \( \delta^{15}\mathrm{N} \) and \( \delta^{2} \mathrm{D} \). Therefore, differences in wintering habitat for the populations are most likely attributed to differences in soil and aridity, as indicated by \( \delta^{15}\mathrm{N} \), and large-scale meteorological processes, as indicated by \( \delta^{2} \mathrm{D} \), between their wintering areas.

Collared flycatchers have previously been shown to exhibit strong natal (Pärt 1990) and breeding-site philopatry (Pärt and Gustafsson 1989). Here, we show that the repeatability of \( \delta^{13}\mathrm{C} \) and \( \delta^{15}\mathrm{N} \) between years was high. The first hypothesis we propose to explain this pattern is that individuals are also philopatric to their wintering grounds. An alternative hypothesis is that individuals from
the same breeding population are utilizing similar winter habitats/food resources every year, but not necessarily at the same location. Natal and breeding site philopatry in collared flycatchers are considered behavioural adaptations minimizing costs of habitat selection (e.g. Pärt 1991, 1994), maximizing reproductive success (e.g. Doligez et al. 2002), and improving competitive ability (e.g. Pärt 1994). The suggested strong winter site philopatry could be beneficial for similar reasons. The year effect in repeatability found for \( \delta^{13}C \) (and near-significant for \( \delta^{15}N \)) suggests that environmental conditions at the wintering site slightly fluctuate from year to year. Such between year variations in isotope ratios may be common for migratory organisms and have previously been reported for black-throated blue warblers breeding in North America (Graves et al. 2002). The lack of repeatability between years in \( \delta^{13}D \) is not surprising given the large between year local and temporal variation in precipitation and evaporation during the molting season, which has a strong effect on \( \delta^{13}D \) variation (see above).

Birds from the same breeding population had more similar isotope ratios in their winter-grown feathers compared to birds from other populations. The difference in isotope ratios between these nearby populations could either be due to: 1) different breeding populations wintering at different locations in Africa (again, we don’t know how far apart these locations are situated), i.e. migratory connectivity is strong, or to 2) breeding population similarities in food and/or habitat utilization without a necessarily strong geographic segregation of the breeding populations at the wintering grounds. One possible way to tease apart the competing hypothesis is to sample individuals at their wintering site. One can then match isotope ratios from winter-grown feathers with geographic locations and habitat information. Veen et al. (2007) compared \( \delta^{13}C \) and \( \delta^{15}N \) in winter-grown feathers of collared and pied flycatchers, from the same breeding populations as in this study, and found that the differences between the two species matched the geographical variation of isotope ratios (measured in feathers of another migrating passerine, the willow warbler *Phylloscopus trochilus*, sampled across Africa (Bensch et al. 2006)) at the two flycatcher species’ presumed wintering grounds. However, they did not find any differences between the two species in \( \delta^{15}N \) but only in \( \delta^{13}C \), potentially suggesting that \( \delta^{13}C \) might be a better indicator of spatial differences, at least at a larger geographic scale. If so, it strengthens the interpretation that the difference we observe in \( \delta^{13}D \) and \( \delta^{15}N \), but not in \( \delta^{13}C \), reflects migratory connectivity on a small spatial scale. Or alternatively, that the difference in \( \delta^{15}N \) reflects habitat or diet differences within the same geographical region without the various breeding populations necessarily wintering in different areas. If so, why birds from the same breeding population would utilize similar habitat or would be specialized on the same food resources remains to be clarified. One possibility is that there is a difference between populations in the quality of their members or that birds from different breeding areas arrive at different times on the wintering grounds – individuals from the later arriving populations or with lower quality members might settle in similar quality territories and thus, have more similar isotope ratios. However, neither time of arrival at the breeding ground, nor individual quality (male and female body size and the condition dependent size of the male’s forehead patch) were related to the isotope ratios. Thus, the patterns observed in this study are not likely caused by differences in arrival time or individual quality.

However, birds from different breeding populations could either genetically or socially inherit their winter habitat/food preference at their natal site explaining the between population differences in isotopic ratios. So far there are no studies indicating within species differences in food preference or specialization on the breeding or wintering grounds and the collared flycatcher also appears to be a generalist insectivorous passerine. In fact, the diet of the collared flycatcher completely overlaps (dietary overlap 98.7%) with the closely related pied flycatcher on sympatric breeding grounds (same populations as in this study; Wiley et al. 2007). Although we currently lack information about the habitat structures where these birds winter. They are all found breeding in very similarly structured deciduous forests (dominated by ash *Fraxinus excelsior*, oak *Quercus robur* and hazel, *Corylus avellana*; pers. obs.).

In addition to breeding population, sex seemed to explain some of the observed variation in \( \delta^{15}N \) (no differences were found for \( \delta^{13}D \) and \( \delta^{13}C \)). Females had an on average 0.67\%\(_{\text{oo}}\) (± SE = 0.29\%\(_{\text{oo}}\)) higher \( \delta^{15}N \) compared to males. This implies that females of a given breeding population winter in more arid (e.g. Shearer et al. 1983) and potentially more stressful habitats (Cormie and Schwarz 1996) than males from the same population (as predicted by the higher \( \delta^{15}N \) values). Alternatively, males and females might be specialized on different food resources or utilize different habitats resulting in differences in stable isotope ratios. These differences in habitat and food choice might be shaped by social dominance, were males dominate females (Greenberg 1986).

The observed difference in isotope ratios in winter-grown feathers between these nearby breeding populations is very intriguing, regardless if the differences are due to variation in habitat/food utilization or strong migratory connectivity on a small spatial scale, and will have many important implications for a range of fields within ecology, conservation and evolutionary biology. The awareness of migratory connectivity and its implication on the annual life cycle of migratory organisms is increasing (e.g. Rubenstein et al. 2002, Webster et al. 2002). Our study highlights that migratory connectivity could very likely be present on a much finer scale than previously considered, implying that collared flycatchers may winter with their breeding-ground neighbours.

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