Inferring the ecology of willow warblers during their winter moult by sequential stable isotope analyses of remiges

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We present a comparison of feather stable isotope (δ¹³C, δ¹⁵N) patterns representing the habitat and diet conditions for two subspecies of willow warblers *Phylloscopus trochilus* that breed in parapatry, but winter in different regions of sub-Saharan Africa. Previous analyses have shown that on average winter moulted innermost primaries (P₁) show subspecific differences in δ¹⁵N values, although individuals show substantial variation for both δ¹³C and δ¹⁵N within the subspecies. We examined whether corresponding variation in the timing of the winter moult, as reflected by consistent intra-wing correlations for individual’s δ¹³C and δ¹⁵N values, could explain some of the previously observed isotopic variation. Further, differential subspecific adaptations to winter precipitation patterns across Africa might result in a variable degree of site fidelity or itinerancy during moult. We found no consistent trend in isotopic values from innermost to outermost primaries, thus inter-individual variation in the timing of moult does not explain the subspecific isotopic variation for P₁. Patterns in wing feather δ¹³C and δ¹⁵N values indicated that 41% of the individuals from both subspecies shifted their diet or habitats during winter moult. Importantly, despite well-documented itinerancy in willow warblers during the winter, 59% of the individuals had feather isotope values consistent with stable use of habitats or diets during winter moult. Repeatability analyses suggest that individuals of both subspecies initiate moult in similar habitats from year-to-year while feeding on isotopically similar diets.

For birds that moult in sub-Saharan Africa, the east–west moisture gradient and differences in precipitation patterns north and south of the equator may make δ¹³C and δ¹⁵N measurements useful indicators of moult provenance (Chamberlain et al. 2000, Evans et al. 2003, Möller and Hobson 2004, Pain et al. 2004). The willow warbler annual cycle includes two complete moults, one after breeding in Eurasia and the other while wintering in Africa (Underhill et al. 1992). If we assume that differences in feather isotope values indicate different isotopic landscapes (isoscapes) or provenance where feathers are grown, a comparison of feather isotope values between both subspecies may reveal differences in climate, habitat, and diet on their respective wintering grounds. Moreover, the δ¹⁵N and δ¹³C feather isoscapes expected for Africa are diverse across the extensive wintering range of willow warblers and so we expected the isotope approach to be sensitive to potential movements of populations within Africa (Hobson et al. 2012).

We know from previous work that δ¹⁵N values differ between west African wintering *trochilus* and east to south African wintering *acredula*, however there is a lot of variation within each subspecies (Chamberlain et al. 2000). Part
of this variation could be caused by individual differences in the location, timing, and duration of their winter moult. The time span between the shedding of the innermost and outermost primary feather is at least six weeks on the winter grounds, but might be substantially longer in slower moult- ing individuals (Underhill et al. 1992, Bensch and Grahn 1993). If the isotopic values are found to change consistently across the wing (from lower to higher values or vice versa), part of the within-subspecies variation in isotopic values as measured for the innermost primary could be caused by a corresponding variation in the timing of moult.

When comparing the subspecies, we predict that because *trochilus* initiates the winter moult in west Africa well after the end of the rainy season we should see δ13C and δ15N values that reflect increasingly arid conditions and the physiological responses to these conditions, i.e. increasing δ13C and δ15N values from the replacement of the innermost to the outermost primary. These patterns are predicted to contrast with *acredula* which initiates moult in east to south Africa at the onset of the rainy season where wet conditions should result in stable or decreasing δ13C and δ15N values across all nine primaries.

Many Palearctic–Afrotropical migrants use an ‘itinerancy’ strategy on their sub-Saharan African wintering grounds, rather than being stationary or territorial (Moreau 1972, Sälewi and Jones 2006). Itinerancy likely reflects the necessity to track highly seasonal resources that increase with the rainy seasons and decrease steadily after the rains end. In the case of itinerant winter residents, individuals may stay in one location to moult or change locations or shift diet in response to changes in resource availability. We know that some willow warblers likely move during their winter moult (Underhill et al. 1992), but it is not known whether this is more common among *trochilus* or *acredula*. We predict greater variability in feather δ13C and δ15N values for *trochilus* as moult is protracted over two months (Underhill et al. 1992) and occurs at the end or after the end of the rainy season where conditions continuously increase in aridity (Janowiak 1988). Due to these increasingly drying conditions, *trochilus* moult may occur over larger isotopic gradients as they track resources following the moisture gradient southeast. In contrast, we predict the patterns of δ13C and δ15N variability across the wing to be less in *acredula* as individuals might show greater site fidelity due to stable resources and moult duration being shorter.

Finally, Bensch et al. (2006) found that there is significant between-year repeatability in δ13C and δ15N feather values, showing that to some extent these birds return to the same geographic region or ecologically similar molting areas from year to year. We used a separate dataset of individuals captured across Sweden in two or more years to compare between-year repeatabilities of feather δ13C and δ15N values with repeatabilities from our wing feathers that represent the beginning (innermost primary) and end (outermost primary) of the moult sequence of the same individual. By comparing the within-year variation (innermost to outermost primary) with the between-year variation (only the innermost primary), we examine to what extent the unexplained between-year variation can be accounted for by within-year variation. Further, low between-year or within-wing (same year) repeatabilities would suggest low winter habitat or site-fidelity and/or switching diet, while on the other hand high repeatabilities suggest high habitat or site-fidelity and/or a similar diet.

### Methods

#### Background and sampling

Passerines typically replace all of their flight feathers and body feathers on an annual basis after breeding and before migration to the wintering grounds or after arrival on the wintering grounds (Svensson 1992, Pyle 1997). Flight feather replacement occurs in a fixed sequence that coincides with the replacement of the entire feather coat (Ginn and Melville 1983). For the willow warbler, the post-breeding moult in adults is quite rapid which can result in periods of flightlessness (Haukioja 1971). In addition, all individuals have a second, more protracted complete moult on their African winter-grounds (Underhill et al. 1992). Both complete moults begin with the innermost primary (P1) and ends with the outermost full-sized primary (P9) (Norman 1990, Bensch and Grahn 1993).

We collected 17 adult willow warblers upon return to Sweden from the wintering grounds in Africa in the spring of 2008 from both sides of the migratory divide, i.e. nine ‘southern’ and eight ‘northern’, for a separate study on the genetic differences of the two migratypes (Table 1). From the same individuals we salvaged the wings for stable isotope analysis. We used the tips for each of the nine full-sized primary feathers from the left wing of each individual for isotopic analyses. From a separate study on the migratypic differences of *trochilus* versus *acredula*, we collected adult willow warbler first primaries from males grown on their wintering grounds in Africa captured on their breeding grounds in Sweden, between 1996 and 2010. Fifty-eight of these individuals were recaptured during this period and were sampled for both δ13C and δ15N (see methods described in Chamberlain et al. 2000 and Bensch et al. 2006).

We collected willow warblers following the ethical guidelines under a permit from Malmö/Lund djurförsöksnämnd, permit number M94-08. Approval for permission for capturing birds came from Swedish Environmental Protection Agency and the Swedish Ringing Centre at the Natural History Museum in Stockholm.

#### Laboratory analysis

We prepared 1.0 ± 0.1 mg of feather material removed from the distal end (the tip) of each primary feather on the left wing from each individual. All samples were loaded into...
tin cups and analyzed for $\delta^{13}C$ and $\delta^{15}N$ via continuous-flow isotope-ratio mass spectrometry (CFIRMS). Specifically, between 0.5 ($\delta^{13}C$) and 1.0 mg ($\delta^{15}N$) of material was combusted online using an elemental analyzer. The resulting CO$_2$ and N$_2$ analyte gas from the samples was separated by gas chromatography (GC) and introduced into a triple-collector isotope-ratio mass-spectrometer via an open split and compared to a pure CO$_2$ or N$_2$ reference gas. Stable-nitrogen and carbon isotope ratios were expressed in delta ($\delta$) notation, as parts per thousand (‰) deviations from the primary standards of atmospheric nitrogen and VPDB (Vienna Pee Dee Belemnite carbonate) standards, respectively. Using previously calibrated internal laboratory C and N standards (powdered keratin, peagrain and gelatin), within-run precisions for $\delta^{15}N$ and $\delta^{13}C$ were $\pm$ 0.15‰.

**Statistical analysis**

We used a general linear mixed model (GLMM) approach to determine the regional differences in $\delta^{13}C$ and $\delta^{15}N$. For this we used the $R$ package nlme (Pinheiro et al. 2013) to test whether our samples demonstrated the previously described regional differences in winter-grown primary feather $\delta^{15}N$ between acerula in the north and trochilus in the south (Chamberlain et al. 2000). Model structure included $\delta^{13}C$ and $\delta^{15}N$ separately (for all nine primaries) as the dependent variables with region as fixed factors and individual as a random factor. Further, we used the same model structure but added the interaction between feather position and region to determine if the variation in $\delta^{13}C$ and $\delta^{15}N$ across the nine primaries differed between regions. To determine if feather isotope values were correlated with primary position across all nine primary feathers from each individual, we calculated a Pearson’s correlation coefficient ($r$) for feather position and each isotope ($\delta^{13}C$ and $\delta^{15}N$) separately. We plotted $r$ for both $\delta^{13}C$ and $\delta^{15}N$ with $\alpha = 0.05$ levels of significance to identify relationships between stable isotope values and feather position. In addition, we plotted the effect size ($r$) with corresponding confidence intervals for each individual to evaluate the strength of the correlation between feather position and feather isotopes ($\delta^{13}C$ and $\delta^{15}N$) and the confidence in interpreting the effect (Nakagawa and Cuthill 2007). Next, we calculated the repeatability or intraclass correlation coefficient (R) using the $R$ package ICC (Wolak 2011), for both isotopes measured across the tips of the tips of P1 (innermost) and P9 (outermost) primary flight feathers. The first and last primaries represent the start and end points of an individual’s moult and thus represent the extremes of the moult period. For this repeatability analysis we used a two-way ANOVA (Shrout and Fleiss 1979, Lessells and Boag 1987), as implemented in the psych $R$ package (Revelle 2012), with feather position as a fixed effect to subdivide the total variability into three components, 1) between-individual, 2) between-feather position, and 3) the residual to determine the intraclass correlation coefficient (Shrout and Fleiss 1979). For comparison, we calculated between-year repeatabilities with a separate Swedish willow warbler dataset (1996 and 2010). Here we used only the first primary (P1) for between-year samples from the same individual. Between-year samples ($n = 58$) allowed us to compare annual differences in habitat use or diet. Finally, we then compared the repeatabilities to determine whether R was lower or higher within the

![Figure 1](image_url)  
**Figure 1.** Effect sizes (correlation coefficient) with 95% confidence intervals for both (A) $\delta^{13}C$ and (B) $\delta^{15}N$ and primary feather position (1 to 9) for willow warbler winter grown feathers in Africa that were sampled in the north and south of Sweden.
same moult episode (P1/P9, wing dataset) compared to the same feather (P1, 1996 to 2010 dataset) in different moult episodes (i.e. between years). All analyses were performed in R ver. 2.14.

Results

We found significant differences in winter-grown primary feather $\delta^{15}$N ($T_{15} = -2.61$, SE $= 0.90$, $p = 0.01$), but not $\delta^{13}$C ($T_{15} = -0.16$, SE $= 0.79$, $p = 0.87$) between southern trochilus that winters in west Africa and northern acredula wintering in east or south Africa. The mean estimates for $\delta^{15}$N values ($\pm$ SD) were 7.4 $\pm$ 1.1‰ for trochilus and 10.0 $\pm$ 2.4‰ for acredula. The corresponding estimates for $\delta^{13}$C were $-21.28 \pm 1.3$‰ for trochilus and $-21.27 \pm 1.4$‰ for acredula. Further, we did not find any differences in the within individual variation across primaries 1 to 9 in $\delta^{15}$N between regions ($T_{15} = -1.24$, SE $= 0.10$, $p = 0.22$), nor did we find any differences in the within individual variation in $\delta^{13}$C between regions ($T_{15} = -1.73$, SE $= 0.12$, $p = 0.08$). Fourteen individual’s showed significant $\delta^{13}$C or $\delta^{15}$N correlations across the primary feathers demonstrating consistent changes in isotopic values from the innermost to the outermost feather. These included three trochilus (all positive) and two acredula (one positive and one negative) for $\delta^{13}$C (Supplementary material Appendix A1, Table A1) and five trochilus (four positive and one negative) and four acredula (three positive and one negative) for $\delta^{15}$N (Supplementary material Appendix A1, Table A2, Fig. 1A, B). Seven of the later individuals revealed significant negative and two positive correlations for $\delta^{15}$N and four showed significant negative and one positive for $\delta^{13}$C; one of these individuals was significant for both isotopes. Of these 14 individuals, inspection of effect sizes and confidence intervals (Supplementary material Appendix A1, Table A1, A2, Fig. 1A, B) demonstrated that only seven of these individuals had confidence intervals that did not overlap zero, supporting that these individuals from both subspecies likely changed their diet or habitats during moult.

Repeatability analyses based on all nine primaries were significant and similar in both the north and south. Repeatability analyses based only on the innermost (P1) and the outermost (P9) primary was significant for $\delta^{15}$N in both regions, but significant for $\delta^{13}$C only in the north (Table 2). For the separate dataset representing between-year captures of the same individuals, repeatability analysis of the innermost primary (P1) for both $\delta^{13}$C and $\delta^{15}$N were significant (Table 3). There were wide confidence intervals with moderate effect sizes for within-year (both for comparing P1 and P9). When we analysed the effect sizes for the repeatability of between-year samples (P1 only) for $\delta^{15}$C, we found a strong effect size with narrow confidence intervals (Fig. 2). The same analyses for $\delta^{15}$N resulted in moderate effect size with wider confidence intervals (Fig. 2).

Discussion

Here we analyzed wing feather stable isotope ($\delta^{13}$C, $\delta^{15}$N) patterns from two willow warbler subspecies with distinctly different wintering areas to investigate population-specific adaptations and ecology during the wintering moult period in Africa, which is an important, but little known part of the annual cycle. We found that in 41% of the individuals the primary feather $\delta^{13}$C and $\delta^{15}$N values changed significantly across the wing from the innermost to the outermost primary feather suggesting a shift in diet, habitat, or location during the winter moult. Contrary to our predictions that trochilus in west Africa would likely show increasing $\delta^{13}$C and $\delta^{15}$N values from the innermost to the outermost feather in response to conditions becoming drier during the moulting period; the observed changes were neither consistent among individuals nor subspecies.

Willow warblers occur in a wide range of habitats across sub-Saharan Africa (Herremans 1997, Saleski and Jones 2006, Jones et al. 2010). The use of these habitats varies temporally with changes in densities recorded throughout the wintering period and across their range, suggesting intracontinental movements or ‘itinerancy’ (Jones et al. 2010). Many species of Palaearctic–Afrotropical migrants, including willow warblers, track the rains associated with the Intertropical Convergence Zone (ITCZ) as it shifts south after the summer rains and then moves back north in the spring (Moreau 1972, Jones et al. 1998, Saleski et al. 2002). Presumably as local habitats dry out and food resources become scare, many individual songbird migrants have to intensify their foraging movements in order to track available resources at different times (Jones et al. 1998, Saleski and Jones 2006).

In west Africa, the drying out occurs between December and March coincident with trochilus winter moult (Williamson 1962, Underhill et al. 1992, Saleski et al. 2004). For trochilus the winter moult of the outermost primary occurs approximately six weeks later than the innermost primary as habitats continuously become dryer. Given these conditions, one might expect $\delta^{13}$C and $\delta^{15}$N values to increase if they follow the general patterns for how these isotopes respond to increasingly arid climate conditions (Rubenstein and Hobson 2004). Although isotopic patterns changed across the wing for six individuals, the direction of change was both towards higher and lower values. Hence, this suggests that the starting date of moult cannot explain the between-individual-variation in isotope values of the innermost primary. Our results indi-

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Figure 2. Effect sizes (correlation coefficient) with 95% confidence intervals given for both $\delta^{13}C$ and $\delta^{15}N$ repeatability analysis for willow warbler winter grown feathers in Africa that were sampled in both the north and south of Sweden. The within-year samples represented by primaries 1 to 9 and between-year samples only primary1.
Acknowledgements – The willow warblers were sampled with ethical permission from Malmö/Lund djurförsökskommittén (M94-08) and permission from Naturvårdsverket and the Swedish Ringing Centre for capturing birds. This project was financed by grants from the Swedish Research Council (to SÅ 621-2007-5930, 621-2010-5584 and SB, 621-2010-5277), a Seedorf Lyne fellowship, Alexander von Humboldt-Foundation (ML), and by operating grants to KAH and LIW from Environment Canada. This is a report from the Centre for Animal Movement Research (CAnMove) financed by a Linnaeus grant (349-2007-8690) from the Swedish Research Council and Lund Univ.

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