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Stable isotopes examined across a migratory divide in Scandinavian willow warblers (*Phylloscopus trochilus trochilus* and *Phylloscopus trochilus acredula*) reflect their African winter quarters

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The C and N isotopes of feathers from two subspecies of willow warblers (*Phylloscopus trochilus trochilus* and *Phylloscopus trochilus acredula*) are isotopically distinct. Our analysis of 138 adult males from 14 sites distributed across Sweden shows that the mean $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of subspecies *acredula* (from latitudes above 63° N) were significantly higher than the mean $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of subspecies *trochilus* (from latitudes below 61° N). The analysed willow warbler feathers had been moulted in the winter quarters and the observed isotopic signatures should thus reflect the isotopic pattern of food assimilated in Africa. The isotopic data observed in Sweden match the cline in morphology, both showing abrupt changes around 62° N. This result agrees with data from ringing recoveries indicating that the two subspecies occupy geographically and isotopically distinct wintering grounds in Africa. Our isotopic data suggest that analysis of stable isotopes of C and N is a promising method to track wintering quarters of European birds that migrate to Africa.

Keywords: stable isotopes; nitrogen isotopes; carbon isotopes; migratory birds; hybrid zone; *Phylloscopus trochilus*

1. INTRODUCTION

Linking breeding and winter quarters of long-distance migratory bird populations has been notoriously difficult, and relatively little is known about whether these populations mix or remain separated on their winter grounds. The traditional method to identify migratory routes and wintering areas of migratory birds has until recently relied exclusively upon analyses of recoveries of ringed birds. Despite a full century of ringing, we only have a fragmented picture of the winter ranges of most long-distance migratory bird species, mainly because of the low probability of obtaining intercontinental ringing recoveries (one out of 10^3 – 10^5 for most small birds). More recent methods, such as orientation experiments using Emlen funnels (Emlen & Emlen 1966) and satellite tracking (ARGOS 1994), will be of much help in revealing migratory directions of local populations, but these techniques are time laborious and expensive, which seriously limits collection of data sets covering larger regions. An alternative and novel approach is to use stable isotope compositions of animal tissues to track migratory patterns. Such a method has recently been applied to neotropical migrant birds (Chamberlain *et al.* 1997; Hobson & Wassenaar 1997; Marra *et al.* 1998), monarch butterflies (Wassenaar & Hobson 1998), Atlantic salmon (Kennedy *et al.* 1997; Harrington *et al.* 1998) and African elephants (Van der Merwe *et al.* 1990; Vogel *et al.* 1990; Koch *et al.* 1995).

In this study, we apply this isotopic approach to two subspecies of willow warblers (*Phylloscopus trochilus trochilus* and *Phylloscopus trochilus acredula*) that are known to have different migratory routes and occupy geographically

distinct winter quarters. A migratory divide is a region in which two populations showing different migratory direction meet; and ringing recoveries of willow warblers in Europe have demonstrated a migratory divide latitudinally in central Scandinavia (Hedenström & Petterson 1984, 1987). In autumn, birds in southern Scandinavia (*trochilus*) migrate south-west, whereas birds in northern Scandinavia (*acredula*) migrate south-east, both heading towards Africa. The available ringing recoveries (figure 1) suggest these birds occupy distinct areas of Africa in the winter. Such differences in migratory direction and migratory distance most likely reflect genetic differences (Berthold 1996; Helbig 1996). Members of both subspecies start moulting all their flight feathers on their African winter grounds in December or early January (Underhill *et al.* 1992). Hence, we test the hypothesis that the isotopic composition of bird feathers collected in the breeding grounds should show a change across the migratory divide between the two subspecies of willow warblers.

Herein, we show that the N and C isotopes of feathers from the two subspecies of willow warblers change abruptly at the presumed location of the migratory divide, i.e. the location for the change in morphology and coloration (Bensch *et al.* 1999). This result most likely reflects the fact that *trochilus* and *acredula* occupy different areas in Africa when replacing their feathers. The results from this study provide supporting evidence for the use of stable isotopes for tracking migratory patterns of birds in general. In addition, the significantly different N and C isotope composition recorded in willow warbler feathers moulted in west and east Africa opens new avenues of research aimed at disentangling migratory routes and wintering areas of Palaearctic–African migratory birds.

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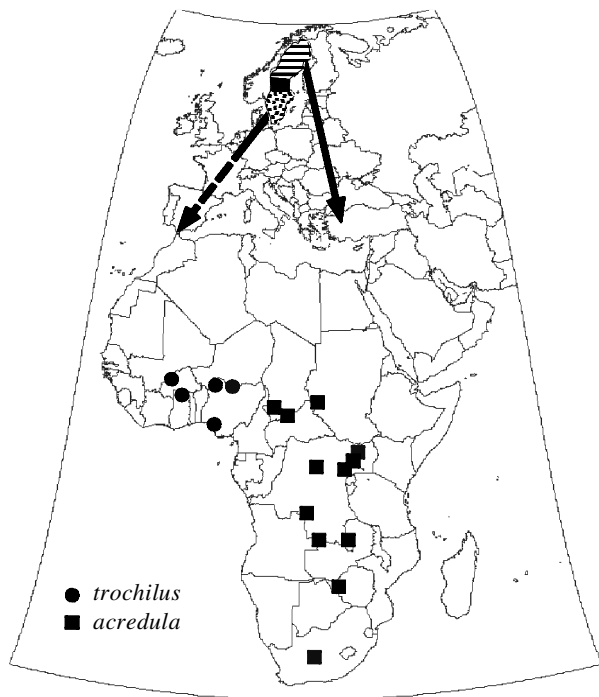


Figure 1. Map of Europe and Africa showing the breeding range of willow warblers of the two subspecies *Ph. t. trochilus* (dotted pattern) and *Ph. t. acredula* (stripped pattern) and their contact zone (black) in Sweden. Arrows show the average migratory direction through Europe of birds supposedly originating from south of 60°N (mean direction $\alpha = 213^\circ$) and north of 63° (mean direction $\alpha = 174^\circ$) (Bensch *et al.* 1999). Also shown is the ringing recoveries of birds ringed as either *Ph. t. trochilus* (circles) or *Ph. t. acredula* (squares).

2. METHODS

(a) *The study species*

In addition to exhibiting a migratory divide we found willow warblers suitable to study for four additional reasons. First, willow warblers moult all their flight feathers twice annually (in winter and summer) (Underhill *et al.* 1992), which means that feathers grown on winter grounds can be collected at breeding sites and vice versa. Because it has been shown that the stable isotopic composition of feathers reflects the food eaten during their growth (Mizutani *et al.* 1990, 1992; Hobson & Clark 1992) it should be possible to use feathers collected on northern breeding sites to determine from approximately where in Africa the birds originated. Second, willow warblers have a large winter range in tropical Africa (Cramp 1992) hence increasing the possibility of detecting geographically consistent isotope patterns. Third, willow warblers are very common with a continuous distribution in Sweden (Cramp 1992) so enough samples can easily be obtained from any specific locality. Fourth, the two subspecies are very similar with extensive morphological overlap, and existing data suggest no differences in feeding behaviour or prey selection (Cramp 1992), which otherwise could have biased the isotopic signals. That the two subspecies are recently derived, is supported by the observation that they lack differentiation at neutral genetic markers (mitochondrial and microsatellites DNA) (Bensch *et al.* 1999).

(b) *Analyses of ringing recoveries*

We used the ringing recoveries reported to the Swedish Museum of Natural History between 1960 and 1998. To identify

whether the ringing recoveries in Africa were representing *Ph. t. trochilus* or *Ph. t. acredula* we used the following criteria. Those birds ringed in Sweden south of 60°N either before 10 May (before the first *Ph. t. acredula* migrates through southern Sweden (Hedenström & Pettersson 1984)) or during June and July were identified as *Ph. t. trochilus* ($n = 5$). Those birds ringed either north of 63°N, or after 1 September in southern Sweden (when the majority of *Ph. t. trochilus* already have departed from Sweden (Hedenström & Pettersson 1984)) were identified as *Ph. t. acredula* ($n = 12$). The subspecies identity of eight birds was ambiguous and therefore excluded from analyses.

(c) *Data collection and analyses of stable isotopes*

In May and June of 1996 and 1997, we sampled feathers of male willow warblers captured on their breeding territories in mist-nets with aid of song playback at 32 sites in Sweden between 55°N and 67°N (Bensch *et al.* 1999). Different primary feathers from the same bird can have different isotopic values reflecting change in diet during growth of individual primaries (Thompson & Furness 1995; Bearhop *et al.* 1999). To avoid these possible complications all samples in this study were from the innermost primary feather on the left wing. In addition, not all willow warblers moult their flight feathers during the winter (Ginn & Mellville 1983). In these analyses we therefore only included feathers from birds in fresh plumage (>95% of examined birds), which apparently had undertaken a complete moult prior to spring migration. We analysed feathers from 138 males representing 14 sites distributed across Sweden for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values (table 1). It is also important to note that the N and C isotope ratios of feather keratin remain fixed after growth (Mizutani *et al.* 1990, 1992; Hobson & Clark 1992). In addition, we routinely analyse a feather standard in our laboratory and there has been no change in the N and C isotopic values of the standard since we began this line of research in 1994; so any processes such as abrasion and deterioration do not affect the isotopic ratio of feathers. Because the N and C isotope ratios of feather keratin do not change after growth they provide a non-invasive method for the study of both recently collected samples and museum specimens (Thompson & Furness 1995).

Isotope ratios for N and C are presented as δ -values where $\delta = (R_{\text{sample}}/R_{\text{standard}}) - 1 \times 1000$, where $R = {}^{15}\text{N}/{}^{14}\text{N}$ and ${}^{13}\text{C}/{}^{12}\text{C}$, respectively. The N and C isotope reference standards are air (N) and Pee Dee belemnite (PDB) (C). Reported uncertainties are the standard error of the mean. N and C isotopic analyses were made on 0.3–0.7 mg of feather material. All N and C isotope values were determined using an online Carlo Erba (Finnigan MAT, Bremen, Germany) and a continuous flow Finnigan MAT 252 mass spectrometer located at Dartmouth College, NH, USA.

N and C isotope values from the Carlo Erba mass spectrometer were corrected by repeatedly analysing a standard (mesquite leaf tissue) during analysis and comparing the isotopic values of these standards with values for the same standard run offline. These corrections typically are -2% for $\delta^{15}\text{N}$ and $+0.6\%$ for $\delta^{13}\text{C}$. In addition, the precision of the isotopic analyses for feather material was determined to be ± 0.3 and $\pm 0.2\%$ for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ (1 s.d., $n = 10$), respectively.

3. RESULTS

N and C isotopic values for individual birds are shown in figure 2 and mean and 1 s.e. values for individual populations are given in table 1. Two results follow from this analysis.

First, the mean (\pm s.e.) $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of subspecies *acredula* from latitudes above 63°N were

Table 1. *N* and *C* isotope compositions of willow warbler feathers from Sweden

sample site			$\delta^{15}\text{N}$		$\delta^{13}\text{C}$		<i>n</i>
latitude	longitude	sample date	mean	s.e.	mean	s.e.	
55°42' N	13°27' E	7–10 May 1996	+7.25	0.62	-20.43	0.91	10
56°15' N	14°00' E	15–17 May 1997	+7.94	0.30	-21.00	0.58	10
58°19' N	14°49' E	28–30 May 1997	+7.20	0.55	-21.00	0.62	10
59°10' N	15°25' E	12–17 May 1996	+6.60	0.40	-21.74	0.51	10
60°24' N	17°58' E	21–22 May 1996	+7.57	0.41	-21.67	0.44	10
61°43' N	17°25' E	26–27 May 1996	+8.76	0.48	-21.59	0.38	10
61°53' N	12°44' E	16–17 June 1996	+9.07	0.68	-21.70	0.52	10
62°02' N	14°03' E	8–10 June 1997	+8.11	0.44	-21.38	0.22	10
62°28' N	17°29' E	28–30 May 1996	+8.72	0.53	-20.08	0.30	10
63°21' N	12°33' E	10–11 June 1996	+10.16	0.84	-20.21	0.54	10
63°29' N	19°42' E	3–4 June 1996	+9.39	0.38	-19.99	0.18	10
65°58' N	16°07' E	6–8 June 1996	+9.78	0.47	-20.17	0.23	10
65°58' N	24°00' E	22–24 June 1997	+10.61	0.66	-20.36	0.31	10
67°13' N	20°48' E	26–27 June 1997	+10.45	0.52	-18.44	1.30	8

+10.1 ± 0.27‰ (*n* = 48) and -19.9 ± 0.26‰ (*n* = 48), respectively. In contrast, the mean $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of subspecies *trochilus* from latitudes below 61° N were both lower (+7.3 ± 0.21‰, *n* = 50 and -21.5 ± 0.29‰, *n* = 50, respectively). Differences between the mean values of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ for the two subspecies were significant (ANOVA: $p < 0.001$ for both $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$). Within the contact zone, between latitude 61° N and 63° N, the mean $\delta^{15}\text{N}$ value was +8.7 ± 0.27‰ (*n* = 40) and mean $\delta^{13}\text{C}$ value was -21.2 ± 0.21‰ (*n* = 40) (figure 2). There was no effect of year of sampling (table 1) for either C ($F_{1,134} = 0.7$, $p = 0.4$) or N ($F_{1,134} = 0.8$, $p = 0.4$) in the ANOVA testing for differences between regions. Similarly, there was no effect of date of sampling (table 1) for either C ($F_{1,134} = 0.04$, $p = 0.8$) or N ($F_{1,134} = 1.9$, $p = 0.2$).

Second, the N and C isotopic data match the cline in morphology for willow warblers in central Sweden (figure 3). Recent work (Bensch *et al.* 1999) has shown differences in size and plumage coloration in willow warblers across the migratory divide in central Scandinavia. The relationship between mean (\pm s.e.) and latitude for two morphological traits (wing length *n* = 32 sites, and body mass *n* = 32), and C and N isotope compositions of willow warblers captured in Sweden is shown in figure 3. The lines in figure 3 indicate the average values for the populations south and north of the identified transition zone (61–63° N), respectively. Note that the differences in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values occur at the same latitudes as the changes in morphological traits. Because the northern birds are larger than the southern birds one could argue that the differences observed in isotopic signatures were a result of size-related food preferences. However, most of the size variation (>80%) is within and not between the subspecies, and there was no significant effect of size (wing length or body mass) on $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ in the ANOVAs testing for differences between regions (all $p > 0.05$).

Based on morphological features, Bensch *et al.* (1999) estimated the transition zone between northern and southern willow warblers to be less than 350 km. Our isotopic data show a geographical change at least as distinct as for the morphological traits. These results

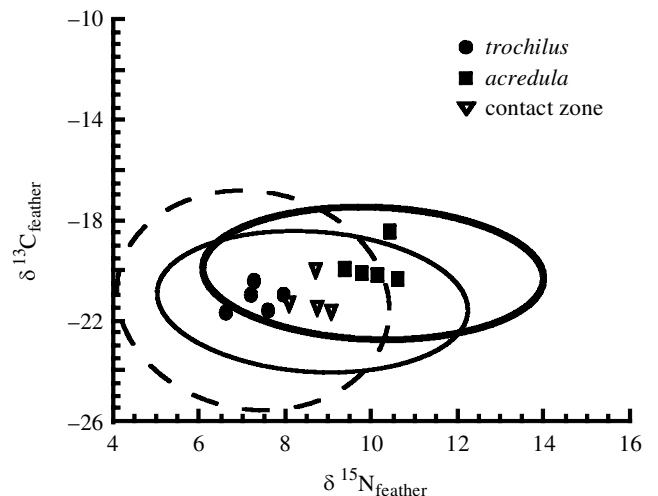


Figure 2. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ mean values (see table 1) in feathers of willow warblers collected in Sweden south of 61° N (circles), between 61° N and 63° N (open triangles), and north of 63° N (squares). The lines represent 95% confidence limits using all data for contact-zone willow warblers, subspecies *acredula*, and subspecies *trochilus*. Dashed line, birds south of 61° N; light solid line, birds between 61° N and 63° N; and heavy solid line, birds north of 63° N.

suggest that the isotopic pattern observed in Scandinavia reflects distinctly different African winter quarters of the two subspecies of Scandinavian willow warblers.

4. DISCUSSION

The willow warbler has a winter range covering most of sub-Saharan Africa, and is found mainly in savannah and forest clearings, avoiding closed evergreen forests (Cramp 1992). The few ringing recoveries suggest that *trochilus* migrates to western Africa and *acredula* migrates to central-eastern Africa (figure 1). However, despite the ringing of 715 000 willow warblers in Sweden up to 1995 (Stolt *et al.* 1997), the few ringing recoveries accumulated so far have not enabled us to locate the position and width of the migratory divide.

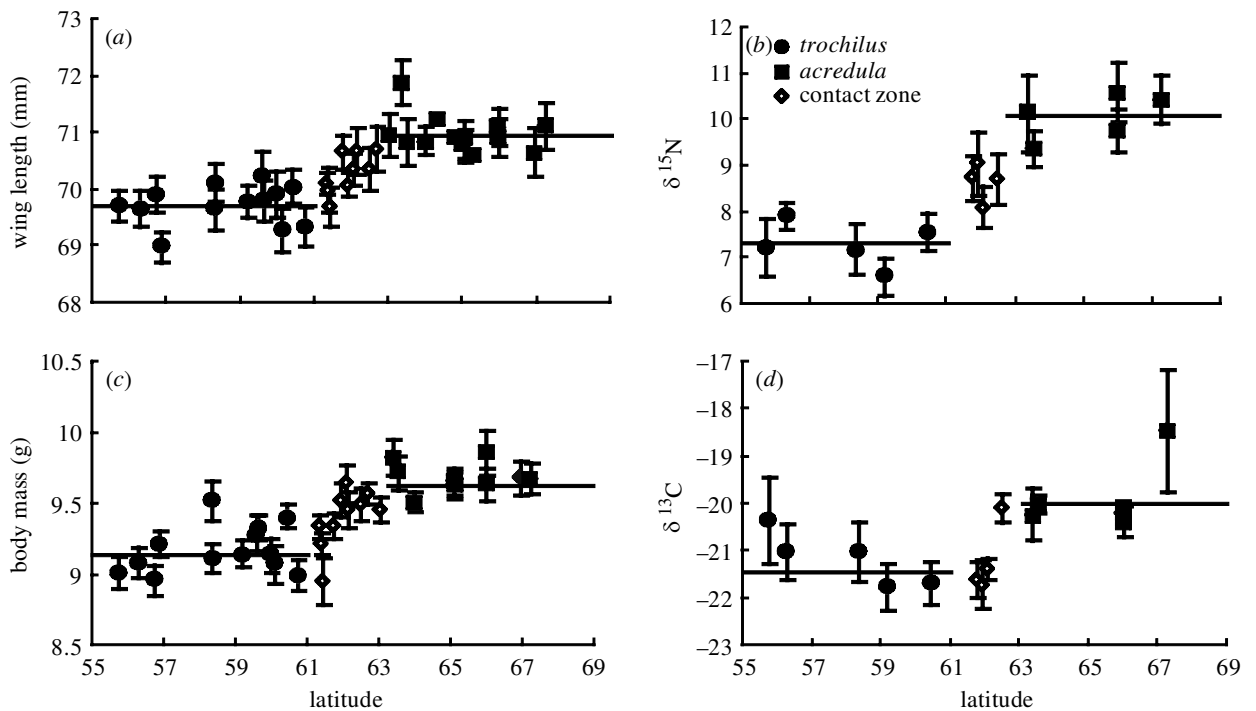


Figure 3. The relationship between mean (\pm s.e.) and latitude for two morphological traits, and C and N isotope compositions of willow warblers captured in Sweden. (a) Wing length ($n=32$ sites), (b) body mass ($n=32$), (c) $\delta^{15}\text{N}$ ($n=14$) and (d) $\delta^{13}\text{C}$ ($n=14$). Mean values for wing length and body size are based on measurements of ten to 20 males per site. The lines indicate the average values for the populations south and north of the identified transition zone ($61\text{--}63^\circ\text{N}$), respectively. Contact-zone willow warblers are presented by open diamonds; subspecies *acredula* by squares, and subspecies *trochilus* by circles.

We found that the C and N isotopes of two subspecies of willow warblers (*acredula* and *trochilus*) changed abruptly at the same latitude (62°N) as the change in morphological traits. It has been shown that: (i) the N and C isotopes of feathers reflect the diet and local environmental conditions where the tissues were grown (Mizutani *et al.* 1990, 1992; Hobson & Clark 1992); and (ii) with the exception of hydrogen isotopes (Chamberlain *et al.* 1997) the isotopic composition of feather keratin is fixed after growth. Thus, the differences in the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values for *acredula* and *trochilus* most likely result from these two subspecies of willow warblers wintering in isotopically distinct areas in Africa, a result which is consistent with rare ringing recoveries mentioned above.

The C and N isotope values of animals are strongly influenced by diet and trophic structure (DeNiro & Epstein 1978, 1981; Minagawa & Wada 1984; Wada *et al.* 1987; Mizutani *et al.* 1990, 1992; Hobson & Clark 1992). During the winter season, willow warblers are primarily insectivorous but the exact trophic structure of the two subspecies is unknown. It is therefore possible that *trochilus* and *acredula* occupy the same geographical region (although this is not supported by ringing recoveries) and the different isotopic signatures reflect intrinsic differences in diet between the two subspecies. We consider this explanation unlikely for the following reasons. First, body size did not correlate with isotopic signatures. Such a correlation would be expected if larger birds were feeding on larger prey types containing different isotope ratios. The lack of a correlation between body size and isotope ratios and the fact that most of the size variation occurs within rather than between subspecies suggest that any difference in trophic structure is not the cause of the

isotopic difference between *trochilus* and *acredula*. Second, we only included males in the analyses so possible differences in prey selection between the sexes (Nyström 1991) cannot have biased the result. Third, we analysed the same feather, the innermost primary, in all birds. This is the first feather the birds are replacing in their moult, and because of the small wing gap at this stage of moult (Bensch & Grahn 1993), they probably can keep their flight performance and usual feeding technique when growing this feather (Hedenström 1998). In addition, start of moult varies only moderately in Africa; early December in Guinea-Bissau, late December in South Africa and early January in Uganda (Underhill *et al.* 1992). Hence, most of the analysed feathers should have been replaced within the same month-long period so that it is unlikely that the isotopic differences between *trochilus* and *acredula* reflect differences in diet at different moulting times.

We, therefore, suggest that the differences between isotopic ratios of *trochilus* and *acredula* primarily result from the two subspecies wintering in isotopically distinct areas. However, without regional isotopic maps of feathers from willow warblers from Africa it is impossible to know exactly where in Africa the two subspecies winter. Since local environmental conditions strongly influence N and C isotopic values it is possible to place constraints on where the two subspecies of willow warblers winter. As mentioned above, willow warblers in Africa are found mainly in savannah and forest clearings (Cramp 1992). The C isotope values of willow warbler feathers are indicative of an ecosystem dominated by C3 African trees, shrubs and grasses (approximately 27‰ (Smith & Epstein 1971; Koch *et al.* 1995)). In addition, the

relatively high $\delta^{15}\text{N}$ values of willow warblers are consistent with a trophic structure originating at base level African plants ($\delta^{15}\text{N}$ of approximately 5 to 3‰) in areas with relatively low mean annual precipitation; less than 500 mm per year (Heaton 1987; Johnson *et al.* 1997). Our isotopic data on willow warblers are, therefore, consistent with the observations that the dominant habitat of willow warblers in Africa is savannah and forest clearings.

We suggest that the higher $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of subspecies *acredula* compared with subspecies *trochilus* reflect the fact that *acredula* winters in an area (East Africa) with a more arid climate and a higher ratio of C4–C3 plants. This argument is based on the following two reasons. First, studies have shown that elevated $\delta^{15}\text{N}$ values of plant (Heaton 1987) and animal tissues (Van der Merwe *et al.* 1990; Heaton *et al.* 1986; Sealy *et al.* 1987; Ambrose 1991; Ambrose & DeNiro 1989; Johnson *et al.* 1997) in Africa occur in areas with relatively low rainfall. This increase in $\delta^{15}\text{N}$ values is presumably the result of ^{15}N enrichment of soil N in dry soils due to higher rates of N loss (Shearer *et al.* 1978) and propagation of this N isotope signal through the food web. The elevated $\delta^{15}\text{N}$ *acredula* is consistent with these N isotope studies. Second, the relatively high $\delta^{13}\text{C}$ in animal tissues reflects, in part, the increased abundance of C4 relative to C3 plants in the ecosystem because C4 plants have higher $\delta^{13}\text{C}$ values (mean approximately –13‰) than C3 plants (mean approximately –27‰) (Smith & Epstein 1971). In addition, C3 plants have higher $\delta^{13}\text{C}$ values in water-limited habitats (Ehleringer & Cooper 1988), which also would result in elevated $\delta^{13}\text{C}$ of animal tissues in arid areas. We, therefore, speculate that the elevated $\delta^{13}\text{C}$ values observed in *acredula* reflect an increase in the ratio of C4–C3 plants in a water-limited environment.

Our interpretation is consistent with regional isotopic studies of elephant bone collagen in Africa (Van der Merwe *et al.* 1990; Vogel *et al.* 1990; Koch *et al.* 1995). In these isotopic studies it was shown that individual populations of African elephants had distinct $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values (Van der Merwe *et al.* 1990; Vogel *et al.* 1990), although there was considerable variability within a given population due to local environmental effects (Koch *et al.* 1995). In general, east and south African elephants had higher $\delta^{13}\text{C}$ values and similar or higher $\delta^{15}\text{N}$ values than west African elephants (Koch *et al.* 1995, fig. 4). These authors (Van der Merwe *et al.* 1990; Vogel *et al.* 1990) also attributed the high $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values to increased aridity and relative abundance of C4 plants.

Our isotopic data agree with the results from ringing recoveries that *trochilus* and *acredula* winter in distinct areas in Africa. However, for birds within the contact zone (those birds between latitudes 61°N and 63°N) in central Sweden it is unclear from the isotopic data whether these birds represent a mix of the two subspecies or whether these birds winter in a different area in Africa. The C isotopic ratios (figures 2 and 3; table 1) suggest that local sites consist either of pure *trochilus* (three sites) or pure *acredula* birds (one site). In contrast, mean N isotopic ratios within these local sites were intermediate between those of *trochilus* and *acredula*, with the mean $\delta^{15}\text{N}$ value differing significantly from the mean of either subspecies ($p < 0.05$). Hence, the N isotopic ratios suggest that the sites in the contact zone either consist of

a mix of birds from the two subspecies, or that the birds in the contact zone winter in an area with a distinct N isotopic signature. The former explanation is consistent with data on morphology, which indicate the presence of linkage disequilibrium between size and coloration within the contact zone (Bensch *et al.* 1999). This explanation is also consistent with the hypothesis that there is a continuous influx into the contact zone of birds from the north (which are larger and have grey-brown plumage), and birds from the south (which are smaller and have green-yellow plumage) (Bensch *et al.* 1999).

Thus, from our isotopic data alone it remains unclear which migratory route is followed by contact-zone willow warblers. Further research using stable isotope signatures from willow warblers breeding in this contact zone will allow us to test whether birds mate randomly or assortatively with respect to migratory direction and winter location (as measured by isotopic signatures). For example, contact-zone birds that show intermediate isotope signatures (supposed hybrids) can be used in orientation experiments directly testing whether they take up an intermediate course of migration in autumn.

5. CONCLUSIONS

In conclusion, the strong correlation between morphological features and stable isotopic ratios of two subspecies of willow warblers, which migrate to geographically distinct areas in Africa, supports the use of stable isotopes as a method for tracking migratory patterns of birds (Chamberlain *et al.* 1997; Hobson & Wassenaar 1997). We, therefore, suggest that the significantly different N and C isotope composition recorded in willow warbler feathers moulted in West and East Africa, respectively, opens new avenues of research aimed at disentangling migratory routes and wintering areas of Palaearctic–African migratory birds in general. The addition of other isotopic ratios, such as δD and $\delta^{87}\text{Sr}$, which have been used elsewhere (Chamberlain *et al.* 1997; Hobson & Wassenaar 1997), when combined with N and C will allow further discrimination of wintering ranges of bird populations, and reduce the potential problem of high isotopic variability due to local ecological shifts (Koch *et al.* 1995). An important task will be to collect data enabling the construction of regional isotopic maps of feather keratin for migratory species in their African wintering grounds, like those under construction for neotropical birds in North America (Chamberlain *et al.* 1997; Hobson & Wassenaar 1997).

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