

A multi-isotope ($\delta^2\text{H}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$) approach to establishing migratory connectivity in Palearctic-Afrotropical migrants: An example using Wood Warblers *Phylloscopus sibilatrix*

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Abstract. Wood Warblers *Phylloscopus sibilatrix* have declined considerably throughout most of their north and western breeding range in Europe but the causes of this decline are unknown. Declines may be related to factors on the breeding grounds, stopover sites and/or wintering grounds. We used multi-isotope ($\delta^2\text{H}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$) measurements of winter-grown feathers of 314 individuals breeding in the Białowieża Forest (E Poland) to infer where they wintered in sub-Saharan Africa over a 4-year period from 2009–2012. We used both aspatial and spatially specific assignment techniques involving a previously developed clustering algorithm related to long-term patterns of precipitation ($\delta^2\text{H}$) and theoretical plant-based isoscapes ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) for Africa. We determined that our breeding population was consistently assigned to the forested region of the Congo basin. Males were more depleted in ^{13}C and ^2H and more enriched in ^{15}N than females suggesting potential sexual habitat segregation on the wintering grounds. We then similarly examined less extensive samples from Wood Warblers breeding in England, Netherlands, Germany, Switzerland and Karelia (NW Russia), and found a similar assignment to the Congo basin. For all sites, males were isotopically distinct from females suggesting sex-specific habitat segregation on the wintering grounds. Our geospatial assignment model now provides a protocol for testing the hypothesis that declining populations winter more in heavily fragmented forests of west Africa compared to the Congo basin. We encourage this approach for the investigation of migratory connectivity in other sub-Saharan Afrotropical migrants.

Key words: stable isotopes, migratory connectivity, wintering sites, deuterium, carbon-13, nitrogen-15, isoscapes, *Phylloscopus sibilatrix*

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INTRODUCTION

It has long been recognized that the effective conservation of migratory species requires a knowledge of where populations travel throughout their annual cycle (Moore et al. 1995, Sherry & Holmes 1995, Webster & Marra 2005). Establishing the linkage between the breeding, wintering and stop-over sites used by individual migratory animals or their populations (hereafter migratory connectivity) is a first step toward conservation

throughout the annual cycle of migratory animals. Conservation efforts directed at only one place or time in the annual cycle will be ineffective if the population is limited by factors occurring elsewhere. Ideally, conservation efforts would be coordinated at the population level so that habitat requirements on breeding, stopover and wintering areas could be met. For migratory birds, such coordination is exceptionally rare and largely restricted to critically endangered populations that move among known and well-defined

locations (e.g. Whooping Crane, *Grus americana*, Lewis 1995). For the vast majority of non-game migratory birds of the world, we simply lack the necessary information on migratory connectivity needed for their effective conservation and much effort has been applied over the last decade in particular to derive more effective methods to track individuals and populations throughout their annual cycles.

In addition to extensive ringing programs (Fiedler 2012) to establish migratory connections, the use of measurement of naturally occurring stable isotopes, primarily in feathers of birds (Hobson 2005, Hobson & Wassenaar 2008), holds promise. Satellite transmitters (reviewed in Meyburg & Meyburg 2009) and smaller light-sensitive geolocators also offer some advantages (Stutchbury et al. 2009, Bächler et al. 2010) but all approaches have their drawbacks (Kelly et al. 2008, Bowlin et al. 2010, Hobson 2011). The transmitters cannot be used on smaller species and use of geolocators requires recapture of birds. Wood Warblers *Phylloscopus sibilatrix*, for example, are too small (body mass < 10 g) for satellite transmitters and are on the borderline for the current crop of geolocators, although there are likely to be rapid advancements in this field. In addition, as they show relatively low inter-annual site fidelity (Wesołowski et al. 2009), geolocators are also not useful.

Recent research using stable isotope markers has focused on their use in assigning origins of birds moulting feathers in Africa because that continent has strong spatial structure in foodweb isotope signatures that are ultimately passed on to birds. In addition, developments in the simultaneous use of multiple isotopes to describe distinct isotopic zones in Africa, based on precipitation and plant-based predicted isotopic patterns or isoscapes, have contributed to a more sophisticated approach to the use of isotopes to infer migratory connectivity (Hobson et al. 2012). We used this approach to investigate migratory connectivity for populations of Wood Warbler.

The Wood Warbler is one of several Palearctic-Afrotropical migrants that breeds in temperate forests of Europe and winters in sub Saharan Africa (Fourage 1968). Almost nothing is known of the species distribution and ecology during the winter period. A long-term ringing effort in Europe has yielded less than ten recoveries from the winter grounds (reviewed in Glutz von Blotzheim & Bauer 1991, Cramp 1992, Urban et al. 1997, Norman 2002). The population of breeding

Wood Warblers showed a steady decline in Europe with numbers halved between 1990 and 2006 (Voříšek et al. 2008). These declines occurred mostly in the northern and western parts of the continent (Burfield & van Bommel 2004), whereas in Central (Flade & Schwartz 2004, Chylarecki & Jawińska 2007, Wesołowski & Maziarz 2009) and Eastern Europe their numbers have fluctuated but did not show declining trends (Burfield & van Bommel 2004). Currently, it remains obscure whether this pattern of change is due to some events in the breeding areas or on wintering grounds or both. Preliminary data from Great Britain (Mallord et al. 2012a,b) and Switzerland (Gerber 2011, Grendelmeier 2011) do not indicate changes in breeding habitat, or production of young as likely causes. Thus, events occurring outside the breeding grounds are likely more important. Establishing patterns of migratory connectivity for populations of Wood Warblers across their breeding range would assist in answering this question. For example, wintering ground effects would be likely if stable populations wintered in different or more secure areas than those of declining populations. Alternatively, if both declining and stable populations wintered in the same area, then factors on the breeding grounds or stopover sites causing differential population declines would be more likely. As a first step to addressing differential population declines of Wood Warblers breeding in Europe, we used multiple ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^2\text{H}$) stable isotope measurements over several years to assign winter origins to a relatively stable population breeding in the Białowieża Forest of Eastern Poland. We investigated the use of a spatially explicit multi-isotope approach to derive a single probability surface of origin for this population. Our specific objectives were to identify the wintering grounds in Africa of a non-declining population, and to investigate if there was evidence for habitat or spatial segregation on the wintering grounds between the sexes. We also examined if there was evidence that earliest arriving birds on the breeding ground derived from different winter quarters. We then followed up that investigation with the less extensive analysis of feathers from elsewhere in Europe, including the UK, Germany, Netherlands, Switzerland and NW Russia (Karelia). The protocols derived here provide a path forward to test hypotheses regarding differential population declines through the derivation of migratory connectivity of this and other Palearctic-Afrotropical migrant songbirds.

METHODS

Study areas and field methods

Wood Warbler feathers were sampled primarily during their breeding season in the Białowieża National Park (co-ordinates of Białowieża village: 52°41'N, 23°52'E) in Eastern Poland. The forest represents a remnant of the vast lowland forests that once covered large parts of temperate Europe (Tomiałojć & Wesołowski 1990, 2005, Wesołowski 1985, 2007). As part of a larger study by Wesołowski and colleagues (Wesołowski et al. 2009, Wesołowski & Maziarz 2009), feather samples were obtained in 2009–2012, in the chiefly upland stands composed mostly of Hornbeam *Carpinus betulus*, Lime *Tilia cordata*, Pedunculate Oak *Quercus robur*, Spruce *Picea abies* and Norway Maple *Acer platanoides*. Data were also collected, in swampy riverine forest, made up mainly by Alder *Alnus glutinosa*, Ash *Fraxinus excelsior* and spruce and in coniferous stands composed of spruce and Scots Pine *Pinus sylvestris* with a admixture of birches *Betula* spp. and some oaks. The majority of males were caught shortly after arrival in mist-nets placed on their territories and using song play back combined with presentation of a dummy of a male Wood Warbler. Females were caught on nests during the second half of the incubation period. All females caught on nests resumed incubation shortly after releasing with no case of nest desertion due to this technique observed. Additionally, in a minority of nests found only at the nestling stage, males and females were caught in mist-nets placed near the nest. Outer rectrices were plucked from all the birds caught and preserved dry. We used dates of egg laying commencement as a proxy of the female arrival dates. The first egg dates were estimated by “counting-back” (for rationale and details see Wesołowski & Maziarz 2009).

Additional feather samples from breeding populations in western Europe and Karelia were obtained from collaborators who used similar capture and sampling techniques to those described above. In the UK, feathers from breeding females were collected within a fragmented wooded landscape on Dartmoor, Devon in south-west England (c. 50°34' N, 4°0' W) in 2012. Woods were dominated by Sessile Oak *Quercus petraea*, with smaller numbers of Silver Birch *Betula pendula*, and a poorly developed understorey, mainly consisting of Rowan *Sorbus aucuparia* and Hazel *Corylus avellana*, which is typical habitat within the species'

range in western Britain (Mallord et al. 2012a). In the Netherlands, feathers from birds breeding in the province of Drenthe (52°51'N, 6°19'E) were collected in 2012–2013, as a part of more extensive study (Bijlsma 2012, 2013). The birds nested there mostly in commercial plantations of Pedunculate Oak, *Larix leptolepis*, Spruce and Scots Pine, intermixed with deciduous trees (Pedunculate Oak, *Rhamnus frangula*, Rowan, *Prunus serotina*), habitat type typical for the Netherlands. The German samples (only males) were collected in Thuringia in 2012, in locations centered on 50°30' N and 11°37'E (K. Evans pers. com.). The Swiss samples were collected in the Jura mountains (c. 47°23'N, 7°45'E) in 2011. The study areas were located on steep, mostly 130 south-facing slopes within large deciduous forests dominated by Beech *Fagus sylvatica* with admixture of oaks and various conifer species (Gerber 2011, Grendelmeier 2011). The Karelian samples origin from birds caught in the vicinity of Ladoga Lake, c. 60°41–46'N, 32°48–56'E, in 2012 by N. V. Lapshin. Characteristics of the study area are given in Lapshin (2005).

Stable isotope analyses

All feathers were cleaned of surface oils in 2:1 chloroform:methanol solvent rinse and prepared for $\delta^2\text{H}$, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis at the Stable Isotope Laboratory of Environment Canada, Saskatoon, Canada. The non-exchangeable hydrogen of feathers was determined using the method described by Wassenaar & Hobson (2003) and using two calibrated keratin hydrogen-isotope reference materials (CBS, KHS). Hydrogen isotopic measurements were performed on H_2 gas derived from high-temperature (1350 °C) flash pyrolysis of 350 ± 10 ug feather subsamples and keratin standards using continuous-flow isotope-ratio mass spectrometry. Measurement of the two keratin laboratory reference materials corrected for linear instrumental drift were both accurate and precise with typical within-run mean $\delta^2\text{H} \pm \text{SD}$ values of $-197 \pm 0.79\text{‰}$ ($n = 5$) for CBS and $-54.1 \pm 0.33\text{‰}$ ($n = 5$) for KHS. All results are reported for non-exchangeable H expressed in the typical delta notation, in units of per mil (‰), and normalized on the Vienna Standard Mean Ocean Water–Standard Light Antarctic Precipitation (VSMOW-SLAP) standard scale.

For $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses, between 0.5 and 1.0 mg of feather material was combusted online using a Eurovector 3000 (Milan, Italy,

www.eurovector.it) elemental analyzer. The resulting CO₂ and N₂ was separated by gas chromatography (GC) and introduced into a Nu Horizon (Nu Instruments, Wrexham, UK, www.nu-ins.com) triple-collector isotope-ratio mass-spectrometer via an open split and compared to a pure CO₂ or N₂ reference gas. Stable nitrogen (¹⁵N/¹⁴N) and carbon (¹³C/¹²C) isotope ratios were expressed in delta (δ) notation, as parts per thousand (‰) deviation from the primary standards: atmospheric nitrogen and VPDB (Vienna Pee Dee Belemnite carbonate) standards, respectively. Using previously calibrated internal laboratory C and N standards (powdered keratin and gelatin), within runs, precisions for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ were better than $\pm 0.15\text{‰}$.

Statistical analysis and assignment to moult origins

We had data for samples collected across multiple years (2009–2012) from our Białowieża sample, and thus we used multivariate analysis of variance (MANOVA) based upon Pillai's trace statistic to test for multivariate ($\delta^2\text{H}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$) isotopic differences among years and sexes within this sample. We modelled isotopic variance using a MANOVA including year and sex as main effects as well as their interaction. Similarly, we compared the isotopic composition ($\delta^2\text{H}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$) of feathers between study areas using a MANOVA model including site and sex as factors, but did not include their interaction as samples at one site consisted of only males and at another site consisted of only females, and thus the interaction was not estimable.

Two separate assignments to moult origin were conducted. First, a conservative aspatial assignment to the isotopic regions of Africa was based on the approach defined by Hobson et al. (2012). This approach assigned individuals to pre-defined isotopic clusters in Africa. Secondly, a new spatially explicit multi-isotope assignments to origin was used by first calibrating previously described isoscapes reflecting a) amount-weighted mean growing-season $\delta^2\text{H}$ in precipitation ($\delta^2\text{H}_p$, Bowen et al. 2005), b) the theoretical spatial $\delta^{13}\text{C}$ distribution of plants in Africa (Still & Powell 2010), and c) a plant $\delta^{15}\text{N}$ isoscape developed by Craine et al. (2009). Calibration of these separate isoscapes into a cluster analysis describing four discrete multi-isotope feather isotope zones in Africa was first reported in Hobson et al. (2012). In brief, Hobson et al. (2012) used parameters from regression of $\delta^2\text{H}$ in feathers of known-origin

($\delta^2\text{H}_f$) against $\delta^2\text{H}_p$ to convert the $\delta^2\text{H}_p$ isoscape of Bowen et al. (2005) for Africa into a $\delta^2\text{H}_f$ isoscape. Calibration of plant $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isoscapes was accomplished by applying a discrimination of +2 ‰ to the $\delta^{13}\text{C}$ isoscape to account for plant-feather isotope discrimination, and a corresponding plant-feather trophic discrimination of +5 ‰ to the plant $\delta^{15}\text{N}$ isoscape (for details see Hobson et al. 2012). We took the additional step of using a digital species' distribution map (BirdLife International 2011) to extract only those cells of each isoscape falling within the overwintering range of the Wood Warbler prior to conducting assignments to origin. We subsequently calculated the mean expected isotopic composition for feathers grown in each cluster by summarizing cells within each isoscape falling within each isotopic cluster using a zonal statistics query of the underlying $\delta^2\text{H}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ isoscapes from which the multi-isotope feather isoscape was derived (Table 1), and compared our sample data to these mean expectations for each bin using multivariate normal probability density functions implemented using the mvtnorm package in R (Genz et al. 2011).

Likely origin for each Wood Warbler was derived using a spatially explicit likelihood-based assignment following Wunder (2007), Hobson et al. (2009) and Van Wilgenburg & Hobson (2011). This provided assignment probabilities associated with a continuous isoscape surface compared to the above-described aspatial binning approach to clusters. However, rather than conducting spatially explicit assignments using univariate probability density functions, we evaluated likelihoods of origin by employing a multivariate normal probability density function (hereafter mvn-pdf) as first described by Royle & Rubenstein (2004).

The mvn-pdf was used to assess the likelihood that a given geo-referenced location (x^i) within the feather isoscape for Africa represented a potential origin as follows:

$$f(x^i y^i | \mu_{\text{HCN}}, \sigma_{\text{HCN}}, \rho_{\text{HCN}}) = \frac{1}{2\pi^{-1/2k} |\Sigma|^{-1/2}} e^{-1/2(y-\mu_x)^T \Sigma^{-1} (y-\mu_x)}$$

where f_x represents the spatially explicit probability density function for x^i representing the location of origin given the feather of unknown origin (y^i) with a measured isotopic composition ($\delta^2\text{H}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$). The expected mean (μ), standard deviation (σ) and correlation (ρ) of $\delta^2\text{H}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ are denoted as HCN subscripts, respectively, for a

Table 1. Isoscape predicted isotopic composition ($\delta^2\text{H}$, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ in ‰) of feathers grown in Africa by isotope clusters created by Hobson et al. (2012).

| Cluster | $\delta^2\text{H}$ | | $\delta^{13}\text{C}$ | | $\delta^{15}\text{N}$ | |
|---------|--------------------|-----|-----------------------|-----|-----------------------|-----|
| | mean | SD | mean | SD | mean | SD |
| 1 | -18.8 | 8.3 | -21.0 | 1.9 | 9.7 | 0.4 |
| 2 | -29.6 | 5.5 | -15.1 | 1.5 | 9.6 | 0.4 |
| 3 | -21.4 | 7.3 | -13.9 | 1.5 | 10.4 | 0.4 |
| 4 | 4.3 | 5.9 | -12.6 | 1.6 | 11.1 | 0.5 |

feather grown at that location. The parameter k represents the number of isotopes. For each location (x^i), estimated mean isotopic composition was estimated directly from raster cells in the calibrated isoscapes for $\delta^2\text{H}$, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Thus, the parameter μ_x^i represents a vector of means for each location (x^i) being considered:

$$\mu = [\mu_{\delta^2\text{H}}, \mu_{\delta^{13}\text{C}}, \mu_{\delta^{15}\text{N}}]$$

Finally, the term $|\Sigma|$ represent the variance-covariance matrix:

$$\Sigma = \begin{bmatrix} \sigma_{\delta^2\text{H}}^2 & \sigma_{\delta^2\text{H}, \delta^{13}\text{C}} & \sigma_{\delta^2\text{H}, \delta^{15}\text{N}} \\ \sigma_{\delta^2\text{H}, \delta^{13}\text{C}} & \sigma_{\delta^{13}\text{C}}^2 & \sigma_{\delta^{13}\text{C}, \delta^{15}\text{N}} \\ \sigma_{\delta^2\text{H}, \delta^{15}\text{N}} & \sigma_{\delta^{13}\text{C}, \delta^{15}\text{N}} & \sigma_{\delta^{15}\text{N}}^2 \end{bmatrix}$$

Diagonal elements in the matrix represent expected variance for the given isotope, whereas off-diagonal elements represent covariance between the denoted isotope pairs. Following Royle & Rubenstein (2004), we assumed that covariance was constant across the isoscapes.

Geographic locations that were consistent with the upper 67% of the spatially explicit probability density maps for each individual were selected and coded as 1; all others were coded as 0 (Hobson et al. 2009). Thus, for each individual being assigned to a moult origin, we obtained one binary map. Finally, we depicted the population likely origin by summing over all individual binary surfaces. All analyses were conducted in the R (v 2.15.1) statistical computing environment (R Core Team 2012). For both the spatial and aspatial assignment approaches, variance-covariance was estimated from the multi-isotope data for our Wood Warbler sample using the mvnml package (Gross & Bates 2011), and spatially explicit assignments also employed functions available in the raster package (Hijmans & van Etten (2012).

RESULTS

Moult origins of Wood Warbler from eastern Poland (Białowieża Forest)

Wood Warbler feathers grown on the Afrotropical wintering grounds from our Białowieża sample showed relatively little inter-annual variation in isotopic composition (Fig. 1). However, MANOVA suggested that year ($F_{9,918} = 2.3$, $p < 0.016$) but not the interaction between year and sex ($F_{9,918} = 1.6$, $p > 0.10$) was significant. The inter-year differences were solely due to variation in male isotope values (Fig. 1). Male $\delta^2\text{H}$ and $\delta^{13}\text{C}$ values varied across years (Pillai's trace ANOVA $F_{3,162} = 3.6$, $p < 0.016$ and $F_{3,162} = 2.6$, $p < 0.055$, respectively). Males and females showed statistically distinct isotopic composition of feathers ($F_{3,304} = 5.9$, $p < 0.001$). Males tended to be more isotopically

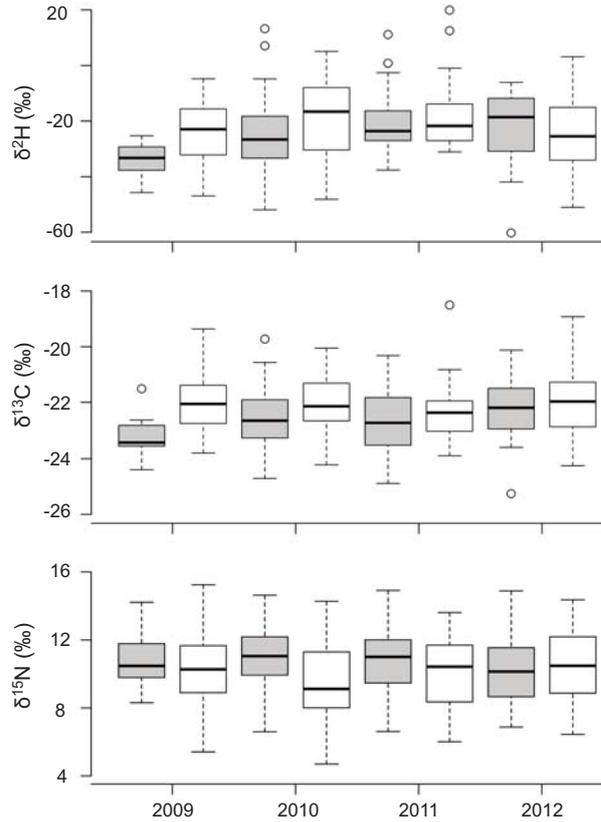


Fig. 1. Inter-annual variation for $\delta^{13}\text{C}$, $\delta^2\text{H}$ and $\delta^{15}\text{N}$ from Afrotropical grown feathers of male (grey) and female (white) Wood Warbler sampled in the Białowieża forest (Poland) in 2009 (7 males, 34 females), 2010 (77 males, 38 females), 2011 (50 males, 34 females), and 2012 (32 males, 42 females). Boxes depict the 25th to 75th percentile range of the data and the horizontal midline within boxes is the median. Whiskers extend 1.5 times beyond the interquartile range, and circles denote outliers.

depleted than females by ~ 2.4 ‰ for $\delta^2\text{H}$ (mean \pm SD: male -23.6 ± 11.7 ‰, $n = 166$; female -21.2 ± 12.5 ‰, $n = 148$), and ~ 0.5 ‰ for $\delta^{13}\text{C}$ (male -22.6 ± 1.0 ‰; female -22.1 ± 1.0 ‰), respectively. In contrast, males were enriched in ^{15}N relative to females by ~ 0.7 ‰ (male $\delta^{15}\text{N}$: 10.8 ± 1.9 ‰; female 10.1 ± 2.2 ‰). Marked birds almost never returned to our study site in the Białowieża Forest the following spring, so repeated isotopic measurements for the same individual were generally not available. Feathers of earlier and later breeding females did not differ in their $\delta^{15}\text{N}$ or $\delta^2\text{H}$ values, but feathers of earlier breeding birds were more depleted in ^{13}C than those of the later ones (Pearson's $r = 0.17$, $n = 140$, $p = 0.044$). Feathers of females commencing laying within 10 days of the yearly median differed significantly ($p = 0.017$) from those commencing later (mean $\delta^{13}\text{C} \pm$ SD: -22.3 ± 1.04 ‰, $n = 128$ and -21.5 ± 1.19 ‰, $n = 21$, respectively).

Moult origins of Wood Warbler across Europe

Multivariate comparison of Wood Warbler samples suggested that they differed isotopically among sites (approximate $F_{6,716} = 5.74$, $p < 0.001$), and between males and females (approximate

$F_{6,716} = 4.38$, $p < 0.001$). Across all sites, females were typically enriched in ^{13}C compared to males by ~ 0.5 ‰ (male $\delta^{13}\text{C}$: -22.1 ± 1.0 ‰; female: -22.6 ± 1.1 ‰), Table 2). Similarly, males were depleted in ^2H than females by ~ 2.2 ‰ (male $\delta^2\text{H}$: -23.3 ± 11.9 ‰; female -21.1 ± 12.4 ‰). In contrast, males were enriched in ^{15}N relative to females by ~ 0.6 ‰ (male $\delta^{15}\text{N}$: 10.7 ± 1.8 ‰; female 10.1 ± 2.1 ‰, Table 2). Across sites, feathers generally showed positive correlations between $\delta^{13}\text{C}$ and $\delta^2\text{H}$ (Pearson's $r = 0.29$ – 0.84 ; see Table 3) except for male Wood Warbler in Karelia, and female Wood Warblers in Switzerland where $\delta^{13}\text{C}$ and $\delta^2\text{H}$ were negatively correlated (Table 3). $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were generally not strongly correlated and correlations were variable between sites (Table 3). The only significant correlations between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were for both females and males in Białowieża, and females in both England and Switzerland (Table 3). Similarly, $\delta^2\text{H}$ and $\delta^{15}\text{N}$ were generally not strongly correlated with the exception of females at Białowieża where $\delta^2\text{H}$ and $\delta^{15}\text{N}$ were negatively correlated (Table 3); however, small sample sizes at some sites (Table 2) prevented stronger conclusions with respect to correlations between isotopes (Table 3).

Table 2. Summary of the multi-isotope ($\delta^{13}\text{C}$, $\delta^2\text{H}$, $\delta^{15}\text{N}$, in ‰) composition of feathers grown on the wintering grounds from female and male Wood Warbler at six sites across the breeding grounds. Only females were sampled in England, and only males in Germany.

| Site | | N | Female | | | n | Male | | |
|--------------------------|-----------------------|-----|--------|------|---------------|-----|-------|------|---------------|
| | | | mean | SD | range | | mean | SD | Range |
| Białowieża (E Poland) | $\delta^{13}\text{C}$ | 148 | -22.1 | 1.1 | -24.3 – -18.5 | 166 | -22.6 | 1.0 | -25.3 – -19.7 |
| | $\delta^2\text{H}$ | 148 | -21.2 | 12.6 | -51.0 – 20.0 | 166 | -23.8 | 11.6 | -60.2 – 13.3 |
| | $\delta^{15}\text{N}$ | 148 | 10.1 | 2.2 | 4.7 – 15.3 | 166 | 10.8 | 1.9 | 6.6 – 14.9 |
| England | $\delta^{13}\text{C}$ | 23 | -22.3 | 1.0 | -24.1 – -19.7 | | | | |
| | $\delta^2\text{H}$ | 23 | -24.7 | 10.2 | -42.5 – -3.3 | | | | |
| | $\delta^{15}\text{N}$ | 23 | 10.0 | 1.7 | 6.0 – 13.3 | | | | |
| Netherlands | $\delta^{13}\text{C}$ | 13 | -22.3 | 0.8 | -24.0 – -21.2 | 30 | -22.5 | 0.8 | -23.9 – -20.8 |
| | $\delta^2\text{H}$ | 13 | -18.6 | 13.5 | -51.3 – -2.5 | 30 | -23.0 | 17.3 | -56.2 – 9.4 |
| | $\delta^{15}\text{N}$ | 13 | 10.3 | 1.0 | 8.75 – 11.8 | 30 | 10.1 | 2.0 | 1.1 – 12.4 |
| Germany | $\delta^{13}\text{C}$ | | | | | 19 | -22.7 | 0.9 | -24.5 – -21.4 |
| | $\delta^2\text{H}$ | | | | | 19 | -31.9 | 12.1 | -59.4 – -6.8 |
| | $\delta^{15}\text{N}$ | | | | | 19 | 11.0 | 2.0 | 7.3 – 14.1 |
| Switzerland | $\delta^{13}\text{C}$ | 15 | -22.5 | 0.9 | -24.2 – -21.2 | 13 | -23.6 | 1.2 | -25.9 – -21.9 |
| | $\delta^2\text{H}$ | 15 | -25.7 | 9.6 | -38.8 – -7.0 | 13 | -28.2 | 9.0 | -44.8 – -10.5 |
| | $\delta^{15}\text{N}$ | 15 | 10.5 | 1.5 | 6.7 – 13.4 | 13 | 11.2 | 1.6 | 9.0 – 14.4 |
| Karelia (NW Russia) | $\delta^{13}\text{C}$ | 5 | -22.4 | 0.8 | -23.3 – -21.4 | 5 | -22.7 | 1.5 | -25.4 – -21.6 |
| | $\delta^2\text{H}$ | 5 | -16.5 | 13.0 | -33.9 – -0.6 | 5 | -36.0 | 10.6 | -45.9 – -19.8 |
| | $\delta^{15}\text{N}$ | 5 | 11.4 | 1.4 | 9.1 – 12.4 | 5 | 9.9 | 2.4 | 7.5 – 13.6 |

Table 3. Pearson correlation between isotopes ($\delta^{13}\text{C}$, $\delta^2\text{H}$, $\delta^{15}\text{N}$) in female and male Wood Warbler feathers grown on the wintering grounds in relation to the breeding sites. * — $p < 0.05$; ** — $p < 0.001$. Sample sizes are given in Table 2. Only females were sampled in England, and only males in Germany.

| Breeding site | | Female | | Male | |
|---------------------|-----------------------|--------------------|-----------------------|--------------------|-----------------------|
| | | $\delta^2\text{H}$ | $\delta^{15}\text{N}$ | $\delta^2\text{H}$ | $\delta^{15}\text{N}$ |
| Poland (Białowieża) | $\delta^{13}\text{C}$ | 0.29** | -0.37** | 0.37** | -0.22** |
| | $\delta^2\text{H}$ | | -0.18* | | -0.06 |
| England | $\delta^{13}\text{C}$ | 0.62** | -0.47* | | |
| | $\delta^2\text{H}$ | | -0.22 | | |
| Netherlands | $\delta^{13}\text{C}$ | 0.56* | -0.16 | 0.68** | 0.16 |
| | $\delta^2\text{H}$ | | -0.06 | | 0.14 |
| Germany | $\delta^{13}\text{C}$ | | | 0.05 | 0.28 |
| | $\delta^2\text{H}$ | | | | 0.38 |
| Switzerland | $\delta^{13}\text{C}$ | -0.03 | -0.62* | 0.48 | 0.27 |
| | $\delta^2\text{H}$ | | -0.08 | | 0.29 |
| Russia (Karelia) | $\delta^{13}\text{C}$ | 0.38 | -0.68 | -0.35 | 0.34 |
| | $\delta^2\text{H}$ | | -0.63 | | -0.63 |

Aspatial assignment to the multi-isotope clusters of Hobson et al. (Hobson et al. 2012) placed all 439 individuals exclusively and unequivocally into Cluster 1 (all likelihoods $> 99.6\%$). Within the wintering range, that assignment region encompasses much of the Congo rainforest and pockets of vestigial rainforest in Côte d'Ivoire and Ghana. The overwhelming assignment to areas consistent with the Congo basin were driven by the isotopic similarity of the samples (means of ~ -22 , -22 , and 10% for $\delta^2\text{H}$, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$, respectively, Fig. 1) with isoscape predictions for Cluster 1 (Table 1). Regionally, these were consistent with isoscape predictions in West African closed canopy forest (Fig. 2A–C). Consequently, spatially explicit assignments predicted that the majority of our Białowieża sample had moult origins consistent with an area between south-eastern Cameroon and eastern Congo, and into the northern Democratic Republic of Congo (Fig. 2D).

A preliminary inspection of assignments to the isoscapes for males versus females revealed little appreciable spatial difference in origins between sexes and so data were pooled from both males and females within sites. Examining the assignment regions for samples from England, the Netherlands, Germany, Switzerland and Karelia suggested similar moult origins to those of birds sampled in Białowieża Forest in eastern Poland,

with the samples largely being consistent with the Congo (Fig. 3A–E respectively). Samples from the Netherlands (Fig. 3B) and Germany (Fig. 3C) tended to be associated with more eastern portions of the assignment region than other samples (Fig 2D and Fig. 3A and D, E). Thus, both the aspatial and spatially explicit assignments to the isoscapes correspond closely with areas of the Congo basin with large tracts of remaining forest cover (Fig. 2D versus Fig. 4), though the spatially explicit assignments did not indicate West Africa as a possible moulting area.

DISCUSSION

Through the exclusive use of endogenous markers sampled on the breeding grounds in the ancient Białowieża forest of Poland, we pinpointed the most probable wintering (moulting) areas of Wood Warblers through four consecutive years for that site. Wood Warblers from Białowieża Forest were consistently assigned to the Congo basin, the area still mostly covered by moist tropical forest. Moreover, those assignment results were found also for several other breeding populations sampled across Europe, albeit for shorter periods of study. Importantly, we have established a methodological approach that can be applied consistently throughout the breeding range of several species of Palearctic-Afrotropical migrants and used to test if there is differential migratory connectivity between stable and declining breeding populations. Specifically, if declining populations of any given species are assigned to (isotopically) different regions of Africa compared with stable populations, much stronger inference on the importance of wintering ground factors in population declines can be achieved.

Wood Warblers breeding in the Białowieża forest showed very consistent isotope values over the four years of the study, with only males displaying slight inter-annual variation. Similar results have been found for other sub-Saharan migrants (Yohannes et al. 2007, Procházka et al. 2008, Hjernquist et al. 2009). This provides support for both consistent patterns of fidelity to the Congo basin wintering grounds and also of stable isotopic conditions in that region of Africa which are characterized by a moist C3 biome. A consistent pattern of fidelity between the Białowieża breeding population and the Congo basin is particularly interesting because ring recoveries at our breeding site are exceptionally rare indicating at

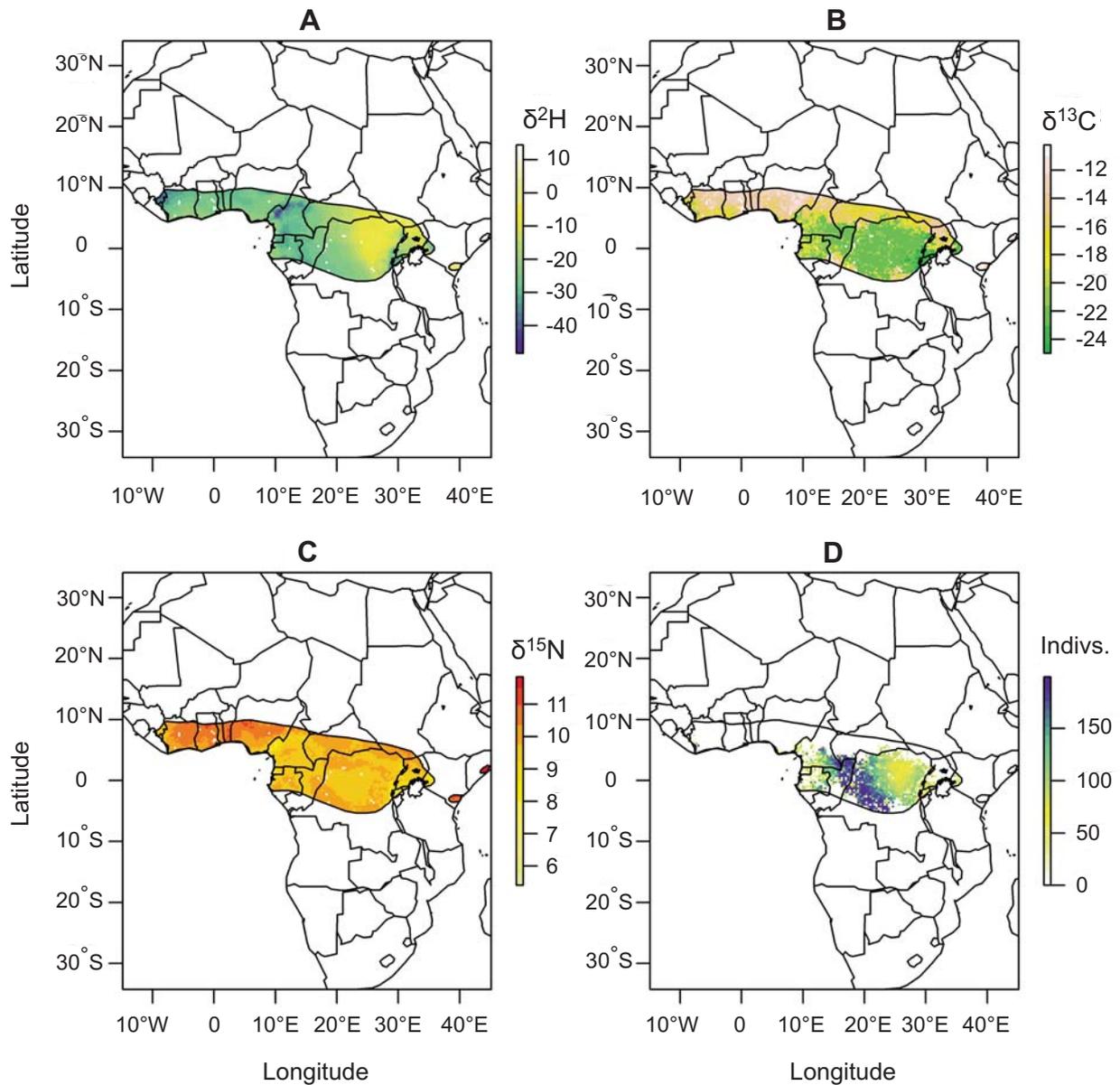


Fig. 2. Predicted isotopic composition of African grown feathers for A) $\delta^2\text{H}$, B) $\delta^{13}\text{C}$, and C) $\delta^{15}\text{N}$, and D) the predicted distribution of moult origins for Wood Warbler sampled in the Białowieża forest ($n = 314$) from 2009–2012. Isoscapes (A–C) were derived by calibration of previously described isoscapes of $\delta^2\text{H}$ in precipitation and theoretical models of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in plants and samples were assigned to the isoscapes using a multivariate likelihood-based approach (see Methods).

least a lack of local breeding philopatry (Wesołowski et al. 2009) or low overwinter survival and high recruitment from elsewhere, which may explain the observed inter-annual isotopic variation observed in males in Białowieża.

Predictions of our spatially specific assignment techniques on the distribution of Wood Warbler moult areas still await ground truthing, but if confirmed, they would suggest the existence of several unforeseen phenomena. Wood Warblers regularly winter and moult in West-Africa

(reviewed in Glutz von Blotzheim & Bauer 1991, Cramp 1992, Urban et al. 1997, Norman 2002), therefore we expected that, similarly as in other European Afrotropical migrants (Zink 1973, Procházka et al. 2008, Zwarts et al. 2009, Korner-Nievergelt et al. 2012, Reichlin et al. 2013), sub-Saharan west Africa, would be a wintering ground of the westernmost Wood Warbler breeding populations. This was not corroborated by our spatially specific assignments, though; the predicted moulting places of westernmost (UK) and

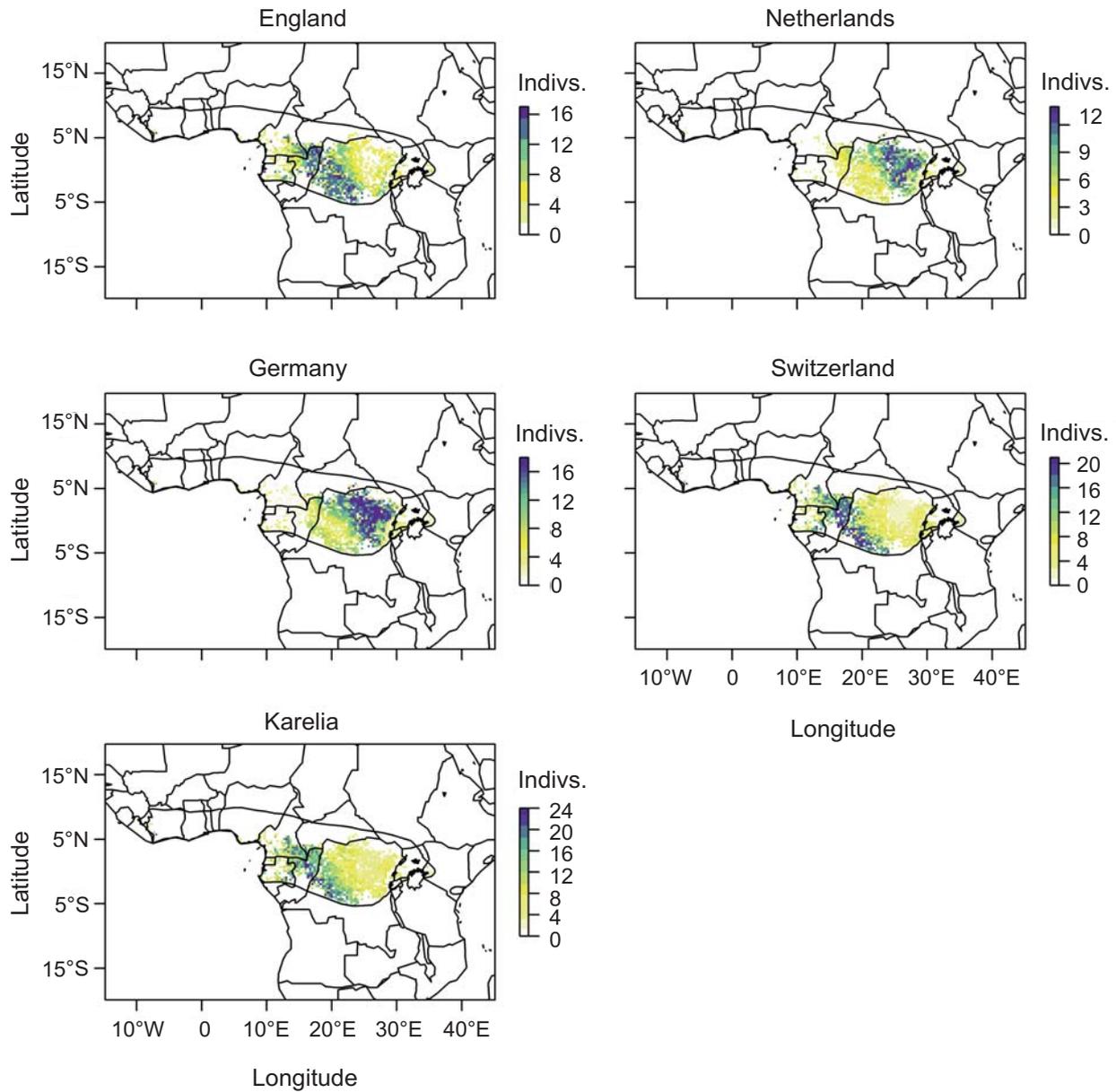


Fig. 3. Predicted geographic distribution of moult origins for Wood Warbler sampled in England ($n = 23$), the Netherlands ($n = 43$), Germany ($n = 19$), Switzerland ($n = 29$), and Karelia, Russia ($n = 11$). Samples were assigned to the calibrated isoscapes presented in Figure 2 (A–C) using a multivariate likelihood-based approach (see Methods).

easternmost (Karelia) breeding birds were situated in the same part of the Congo basin. This leaves an open question, from which parts of the breeding range come the birds wintering in W Africa. Furthermore we expected, that, differences between intensity of human landscape transformation (heavily deforested West Africa vs. less affected Congo basin) could be responsible for contrasting population patterns. This was not confirmed either, wintering areas of UK (declining) and Białowieża (fluctuating) breeding

birds were assigned to the same parts of the Congo basin. So these dissimilar population trends could be rather due to contrasting environmental conditions met by the birds using different migratory routes to reach the same wintering area. It is also possible, that most Wood Warblers winter nowadays in that large tract of forest compared to their known use also of forest fragments of Côte d'Ivoire and Ghana (using only the aspatial cluster analysis approach, Cluster 1 where 99% of all birds were assigned, corresponds to

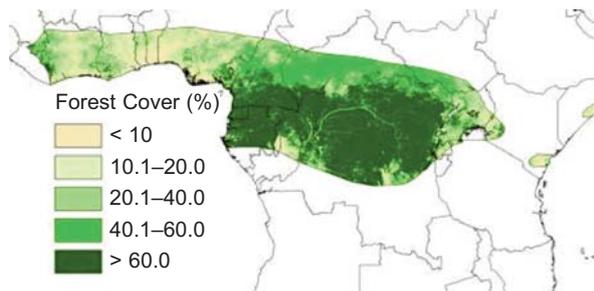


Fig. 4. Percent forest cover (de Fries et al. 2000) within the described wintering range of the Wood Warbler.

these forests as well as the Congo). Possibly, much of the decline in the species has occurred among birds that at one time wintered in the more western portions of their wintering range, but the surviving populations are now heavily biased to the Congo. However, we consider the hypothesis of strong connectivity between both eastern and western European breeding populations of Wood Warblers and the Congo region to still require further testing using much more extensive sampling throughout Europe and also by comparing them with multi-isotope values of feathers of birds caught on different wintering grounds in Africa. A complementary approach, analyses of stable isotopes in Europe-moulted feathers, taken from (juvenile) Wood Warblers upon their arrival on the wintering grounds, could provide information on birth places of birds wintering in different parts of Africa.

We found a weak but consistent difference between the isotopic composition of feathers of male and female Wood Warblers. While both sexes were strongly assigned to the Congo basin, we suggest that this isotopic difference may reflect different spatial or habitat use by males and females. Previous studies have shown evidence for winter habitat segregation among the sexes and age groups of migrant songbirds with the most well-known example being American redstarts *Setophaga ruticilla* wintering in coastal mangrove and shrub habitats of Jamaica (Marra et al. 1998). Like these redstarts, the slightly lower $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values for male Wood Warblers is consistent with males and early arriving females occupying more mesic habitats but again, these isotopic differences between the sexes were relatively small and so no strong conclusions can be drawn. Alternatively, males could be wintering closer to the breeding grounds as documented in other migrant songbirds (Cristol et al. 1999, Jenkins & Cristol 2002, Guillemain et al. 2009).

Several factors contributed to the success of our stable isotope approach for evaluating the wintering grounds of the Białowieża breeding population of Wood Warblers and those from other regions. First, it has long been recognized that a multi-isotope approach will typically increase the resolution of assignment over the use of a single isotope especially if those additional isotopes are related to known underlying spatial structure of foodwebs (Inger & Bearhop 2008, Hobson 2011). This is because such isotopes often respond differently to biogeochemical mechanisms resulting in isotopic discrimination. In the case of the stable isotopes of H, C and N, while these can covary to some degree, each being sensitive to ambient temperature, humidity and rainfall, they also are driven by other related and unrelated mechanisms. For example, $\delta^2\text{H}$ values in foodwebs is dependent on isotopic signatures of source waters as well as amount of precipitation and ambient temperature. Stable-carbon isotope values are dictated largely by plant responses to ambient temperature and moisture via photosynthetic pathway and water-use efficiency mechanisms and $\delta^{15}\text{N}$ values are further influenced by N-fixation processes and soil nitrate signatures in addition to ambient climate (Clark & Fritz 1997, Craine et al. 2009, Still & Powell 2010). Thus while feather $\delta^2\text{H}$ and $\delta^{13}\text{C}$ values were correlated, likely due to similar isotopic discrimination mechanisms related to plant water use and retention, those of $\delta^2\text{H}$ and $\delta^{15}\text{N}$ were not. The combination of all three isotopes thus provided a strong multivariate signal corresponding to physiognomic conditions highly correlated with moist forest represented almost exclusively by our derived Cluster 1. Because Wood Warblers have such a strong association with this habitat on the wintering grounds, our isotopic association proved to be unambiguous. Species whose populations are less restricted to forest or other distinct isotopic regimes (e.g. C4 savannahs) or who use biomes characterized by high isotopic variability such as wetlands or agricultural regions will be more challenging to assign to moult origin.

In their analysis of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in feathers of Wood Warblers breeding in the UK and Germany in 2009, Evans et al. (2012) found isotope values similar to ours (mean $\delta^{13}\text{C}$: -23.4‰ , mean $\delta^{15}\text{N}$: 10.5‰ , $n = 72$). Unfortunately, these authors did not measure $\delta^2\text{H}$ values and did not attempt spatial assignment in Africa using a combined multi-isotope approach. Like many

previous isotopic studies for migrants to Africa (Hobson et al. 2012) inferences made by Evans et al. (2012) were based entirely on the type of habitat occupied during feather growth. Our derivation of a spatially explicit assignment tool based on known and predicted feather isoscapes for Africa demonstrate how feather isotope data can be used more effectively.

As a species that is clearly associated with closed-canopy forest both on the breeding and wintering grounds, survival of the Wood Warbler will be strongly associated with retention of large forest tracts and in sub-Saharan Africa. This is a daunting task with current high deforestation rates in the Congo basin and elsewhere in the wintering range of this species (Zhang et al. 2006). However, recent observations in SW Ghana (D. Sheehan, personal comm.) showing that Wood Warblers are more adaptable in their habitat use, that they can apparently overwinter also in cultivated lands provided they contain tall emergent trees, gives some more hope. We encourage future studies to capitalize on the isotope methods we have developed here and elsewhere (Hobson et al. 2012) for Africa to establish patterns of migratory connectivity for populations of this and other similar migrant species.

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STRESZCZENIE

[Wykorzystanie analizy wieloizotopowej ($\delta^2\text{H}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$) dla ustalenia powiązań migracyjnych Palearktyczno-Afrotropikalnych migrantów na przykładzie świstunki leśnej]

Na wielu obszarach w północno-zachodniej części zasięgu obserwuje się znaczące spadki liczebności świstunki leśnej, a przyczyny tych spadków wciąż nie są znane. Mogą one wynikać z niekorzystnych zmian na lęgowskich, w miejscach postoju na trasie wędrówki i/lub na zimowiskach. Poznanie przyczyn spadku liczebności oraz skuteczna ochrona gatunku wymaga — w pierwszej kolejności — ustalenia tras wędrówek i miejsc zimowania ptaków. Współcześnie dostępne metody badań migracji ptaków (np. nadajniki satelitarne, geolokatory) posiadają ograniczenia nie pozwalające na zastosowanie ich u gatunków takich jak świstunka — ptaków niewielkich rozmiarów (o masie ciała < 10 g), bardzo rzadko powracających na lęgi w te same miejsca. Metoda polegająca na jednoczesnej analizie zawartości kilku trwałych izotopów w piórach, które wyrosły na zimowiskach, a zebrane zostały od ptaków przebywających na lęgowskich, stwarza takie możliwości. W pracy przedstawiamy wyniki badań, których celem było ustalenie miejsc

zimowania w tropikalnej Afryce świstunek pochodzących ze znanych miejsc lęgowych w Europie. W próbkach piór z zimowisk, pobranych od 314 osobników gniazdujących w polskiej części Puszczy Białowieskiej w latach 2009–2012, oznaczyliśmy zawartość izotopów wodoru, węgla i azotu ($\delta^2\text{H}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$). Pozwoliło to określić obszary ich potencjalnych zimowisk w subsaharyjskiej Afryce w kolejnych latach. Przyporządkowania do obszarów dokonaliśmy przy użyciu zarówno niespecyficznych jak i specyficznych metod przypisania przestrzennego. Metody te opierają się na algorytmie grupowania, wykorzystującego informacje pochodzące z długoterminowych modeli rozmieszczenia opadów ($\delta^2\text{H}$) i teoretycznego rozmieszczenia izolinii roślinności ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) w Afryce. Zawartość izotopów ($\delta^2\text{H}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$) w wyrosłych na zimowiskach piórach świstunek leśnych gniazdujących w Puszczy Białowieskiej wskazuje na to, że corocznie pierzyły się one w lasach na terenie basenu Konga. Pióra samców zawierały mniej ^{13}C i ^2H ale więcej ^{15}N niż pióra samic (Fig. 1), co sugeruje pierzenie w różnych siedliskach (obszarach). Podobne wyniki otrzymaliśmy analizując tymi samymi metodami mniej obfite próbki piór świstunek gniazdujących w innych częściach Europy: Anglii (23), Holandii (43), Niemczech (19), Szwajcarii (29), Karelii (10, północnozachodnia Rosja) (Tab. 2, Fig. 3). Skład izotopowy piór wskazywał, że również te ptaki pierzyły się w basenie Konga, a samce i samice zmieniały upierzenie w odmiennych warunkach. Uważamy, że przedstawiony w pracy model rozmieszczenia geograficznego i środowiskowego zimujących świstunek może być punktem wyjścia do testowania hipotezy, że ptaki pochodzące z populacji zmniejszających liczebność zimują częściej na znacznie silniej wylesionych terenach zachodniej Afryce niż w basenie Konga.