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ORIGINAL ARTICLE

# Quantifying the non-breeding provenance of staging Ruffs, *Philomachus pugnax*, using stable isotope analysis of different tissues

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**Abstract** International conservation efforts for migratory populations are most effectively based on quantification of the geographical linkages between wintering, staging, and breeding areas, patterns that may not remain constant in times of global change. We used stable isotope ($\delta^{13}$C, $\delta^{15}$N, and $\delta^{2}$H) measurements of different tissues representing distinct periods of dietary integration to quantify the non-breeding provenance of a threatened staging population of Ruffs *Philomachus pugnax*. In 199 staging Ruffs captured in 2012 during northward migration in the Netherlands, we compared the multi-isotope patterns of feathers grown at wintering grounds, with the $\delta^{13}$C and $\delta^{15}$N profiles of blood cells and plasma representative of staging areas. Few birds had the $^{13}$C-depleted and $^{15}$N-enriched feathers suggestive of wintering quarters in European agricultural areas. Most Ruffs had higher feather $\delta^{13}$C values, suggesting that they wintered in sub-Saharan Africa. Feather $\delta^{2}$H values were not informative due to the overlap of values from European and African moulting sites. Blood cell $\delta^{13}$C and $\delta^{15}$N values indicated that sub-Saharan Ruffs fuelled on low trophic-level foods in habitats dominated by C3 terrestrial or freshwater aquatic primary production, e.g. the rice fields in Africa or the Mediterranean. Stable isotope ratios in plasma suggested that Ruffs made stopovers in southern European agricultural areas. Stable isotopes thus enabled assessments of wintering origin in large numbers of birds. We further propose that conservation measures to protect Ruffs must include the adequate management of sub-Saharan wetlands, based on a better understanding of the role of human-made rice fields for fuelling birds.

**Keywords** *Philomachus pugnax* · Wintering · Spring migration · Migratory connectivity · Stable isotopes · Shorebirds

## Zusammenfassung

### Quantifizierung der Herkunft rastender Kampfläufer *Philomachus pugnax* mit Hilfe der stabilen Isotopen-Analyse von unterschiedlichen Gewebetypen

Internationale Schutzbemühungen für ziehende Populationen sind am effektivsten, wenn sie auf der Quantifizierung der geografischen Verbindungen zwischen Überwinterungs-, Rast- und Brutgebiet basieren, da in Zeiten des globalen Wandels deren Muster nicht konstant bleiben. Mit Hilfe stabiler Isotopen-Messungen ($\delta^{13}$C, $\delta^{15}$N und $\delta^{2}$H) aus verschiedenen Gewebetypen, die unterschiedliche Perioden der Nahrungsaufnahme repräsentieren, um die Herkunftsgebiete einer bedrohten Rastpopulation von

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Kampfläufern *Philomachus pugnax* zu quantifizieren. 2012 wurden dazu während des Heimzuges 199 rastende Kampfläufer in den Niederlanden gefangen. Wir verglichen die Multi-Isotopen-Muster der Federn, die in den Überwinterungsgebieten gewachsen waren, mit den $\delta^{13}C$ und $\delta^{15}N$ Profilen der Blutzellen und Plasma, die typisch sind für die Rastgebiete. Einige Vögel hatten $^{13}C$-verminderte und $^{15}N$-angereicherte Federn, was auf Überwinterungsgebiete in europäischen Agrargebieten hinweist. Die meisten Kampfläufer hatten höhere $\delta^{13}C$ Werte in den Federn, was auf eine Überwinterung in Afrika südlich der Sahara hindeutet. Federn mit $\delta^{2}H$ Werten lieferten keine Informationen aufgrund der Überlappung der Werte aus Europa und afrikanischen Mausergebieten. $\delta^{13}C$ und $\delta^{15}N$ Werte der Blutzellen deuten darauf hin, dass sich südlich der Sahara überwinternde Kampfläufer mit Nahrung niedriger Trophiestufen „auftanken“in Habitaten, dominiert von C3 aus terrestrischer oder Süßwasser-Primärproduktion, z. B. Reisfelder in Afrika oder im mediterranen Raum. Die Verhältnisse stabiler Isotope im Plasma legen nahe, dass Kampfläufer in Agrargebieten Südeuropas rasten. Stabile Isotope erlauben daher einen Rückschluss auf die winterlichen Herkunftsgebiete bei einer großen Anzahl von Vögeln. Darüber hinaus sollten Maßnahmen zum Schutz von Kampfläufern ein adäquates Feuchtgebietsmanagement in der Subsahara beinhalten, basierend auf einem besseren Verständnis der Rolle von Reisfeldern für die Fettdeposition für den Zug.

## Introduction

Migratory birds occur all over the globe, travelling from one biome to another to take advantage of favourable conditions seasonally (Alerstam 1990; Newton 2008). Along their flyways these widely ranging species experience increasing human pressures on their environment, which often contribute to population declines (Baker et al. 2004; Wilcove and Wikelski 2008; Runge et al. 2015; Piersma et al. 2016). Effective conservation measures should be based on information of migratory connectedness of populations, taking into consideration the full life cycle requirements of species and critical bottlenecks or sites (Webster et al. 2002; Piersma 2007; Taylor and Norris 2010; Iwamura et al. 2013). Unfortunately, such information is often incomplete or lacking for migratory birds (Iwamura et al. 2013; Gilroy et al. 2016; Márquez-Ferrando et al. 2014). This hampers the development of effective demographic monitoring and international conservation strategies (Iwamura et al. 2014). Species using diffuse networks of inland freshwater habitats, or those with poorly described migratory pathways, provide the greatest challenges for the conservation of wetland-associated species (Skagen et al. 2003).

Numerous tools are currently available to quantify geographic linkages of migrating populations. The use of individual ring recoveries, recaptures, or resightings (Thorup et al. 2014) is presently augmented by the use of modern tracking devices including satellite telemetry (e.g. Gill et al. 2009) and geolocation (e.g. Tøttrup et al. 2012; Ouwehand et al. 2015). For cases where birds move between sites with food webs showing distinct isotopic compositions, a complementary approach has been the use of stable isotope measurements in animal tissues (Hobson 1999, 2005; Hobson and Wassenaar 2008). Interpretation of stable isotope analyses rely on the existence of predictable spatial patterns in isotopic ratios of substrates that vary due to a variety of biogeochemical processes. These processes ultimately shape isotopic “landscapes” or “isoscapes”, with such variations being passed on to higher trophic levels (e.g. Catry et al. 2016; Christianen et al. 2017). These isoscapes may serve as a basis to examine retrospectively the geographical origin of sampled animal tissues, hence the origin and movements of organisms themselves (Hobson 1999, 2005; Hobson and Wassenaar 2008; Catry et al. 2016). Isotopic values of tissues incorporated from the environment at the time of growth are maintained until the tissue is renewed or replaced. Based on a careful choice of tissues, stable isotope analyses provide a complementary and cost-effective method to monitor migratory linkages over time (Hobson and Wassenaar 2008; Dietz et al. 2010; Yerkes et al. 2008).

Here we used stable isotope measurements to quantify the current geographical linkages of an endangered staging population of Ruffs, *Philomachus pugnax*, migrating along the East Atlantic Flyway (Verkuil et al. 2012; Schmaltz et al. 2015). Ruffs are lek-breeding shorebirds, well known for their strong sexual dimorphism and the extravagant nuptial plumage of males (van Rhijn 1991). The species is strongly associated with inland wetlands habitats, whose migration typically takes place on a broad front between northern Eurasia where they breed, and Africa and India where they winter (Cramp and Simmons 1983). Ruffs along the East Atlantic Flyway stage in the Netherlands during spring migration then to move on to breeding areas at the sub-arctic and arctic latitudes of Eurasia (Jukema et al. 2001), before returning to the floodplains of the Senegal Delta, Inner Niger Delta in Mali and Chad Basin in winter (Zwarts et al. 2009). A few thousand males, however, do not cross the Sahara and winter instead in northwest Europe (Prater 1973; Castelijns 1994; Gill et al. 1995; Devos et al. 2012; Hornman et al. 2013), Iberia (Hortas and Masero 2012), and Morocco (Qninba et al. 2006—Fig. 1). Jukema et al. (2001) suggested that European winterers are the earliest arriving birds at the Dutch staging areas in spring, followed by sub-Saharan wintering males and late females.

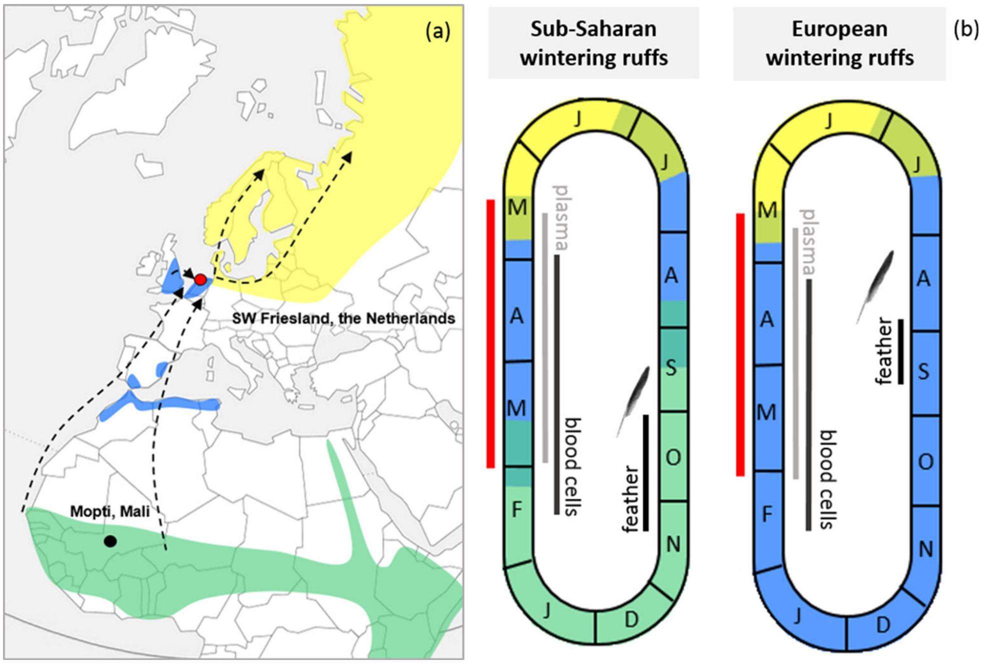

**Fig. 1** **a** Distribution range of Ruffs with the location of the collection sites of tissue samples. The wintering areas of Europe are displayed in *blue*, wintering range in sub-Saharan Africa is in *green* and the breeding range of the species in *yellow*. *Dashed arrows* indicate the East Atlantic Flyway corridor. Staging birds were captured on their main spring staging area in southwest Friesland, the Netherlands. One wintering female was sampled in Mopti, Mali. **b** Annual cycle of Ruffs wintering in sub-Saharan Africa and in Europe, with respective time window of isotopic integration for plasma, blood cells, and feathers, relative to the period of spring capture on the staging site in Friesland (indicated by the *red stripe*). *Colour code* is the same as for **a** (colour figure online)

The staging population in spring in the Netherlands has shown a severe decline since the late twentieth century. Of the 20,000 birds counted during peaks of spring migration on communal roosts in the 1990s, fewer than 5000 remained 15 years later (Verkuil et al. 2012; Schmaltz et al. 2015). The decline may at least partially be explained by a redistribution towards Eastern Europe (Rakhimberdiev et al. 2010; Verkuil et al. 2012). In parallel, however, low female survival, perhaps linked to greater exposure to heavy hunting pressures in Mali than males, could have severe negative consequences for this population (Schmaltz et al. 2015). In sub-Saharan Africa, the control of the hydrology of the Senegal River, and partly also the Niger River, enabled the development of irrigated agriculture, but greatly disrupted the natural dynamics of the floodplains on which Ruffs and many other wintering birds relied on traditionally (Zwarts et al. 2009). Within continental Europe and the Mediterranean, the anthropogenic pressures on natural wetlands continues along with associated intensive land-use for agriculture. Birds are more and more exposed to man-made wetlands, such as ricefields north and south of the Sahara that might constitute at least temporarily, favourable foraging habitat for numbers of waterbirds (Wymenga and Zwarts 2010; Sánchez-Guzmán et al. 2007).

In the context of landscape changes occurring along the entire flyway, we assessed the current wintering quarters and northward migratory patterns of Ruffs using the Netherlands as their main staging area. We inferred wintering quarters of Ruffs from multi-isotopic values ($\delta^{13}$C, $\delta^{15}$N, $\delta^{2}$H) in their 9th primary feather, which is replaced during early winter on their final wintering destination (Pearson 1981; Koopman 1986; OAG Münster 1998). As a metabolically inert tissue, feathers “lock in” the spatial isotopic signals at time of growth (Hobson and Clark, 1992; Bearhop et al. 2002). Unlike feathers, blood is continuously renewed, and in medium-sized shorebirds like Ruffs a $^{13}$C turnover rate will be about 2 weeks for plasma to a month for blood cells (Evans Ogden et al. 2004; Klaassen et al. 2010). This way, we inferred current and recent refuelling areas for arriving Ruffs in the Netherlands by comparing $\delta^{13}$C and $\delta^{15}$N measurements in plasma and blood cells separately. Using these results, we examined the proposed tendency for European wintering birds to arrive at the Dutch staging area before the birds from sub-Saharan wintering areas.

## Materials and methods

### Field methods

Ruffs were captured during spring migration in 2012 at our study site, the core of their staging area in the province of

Friesland in the north of the Netherlands. The area consists of agricultural grasslands intensively managed for dairy farming along the shore of Lake IJsselmeer between the villages of Makkum (53°3.37′N, 05°24.19′E) in the north, Laaksum (52°51.15′N 05°24.77′E) in the south, and It Heidenskip (52°56.93′N, 05°30.11′E) in the east (Schmaltz et al. 2016). During their stay, Ruffs feed on soil invertebrates and above-ground insects in the grasslands, while they rest along shorelines and scattered inland wetlands during midday and at night (Verkuil and de Goeij 2003; Schmaltz et al. 2016; Onrust et al. 2017). Between 10 March and 15 May 2012, 199 adult Ruffs (187 males and 12 females) were caught using traditional clap nets ("wilsternets", Jukema et al. 2001). The low numbers of females in our sample is explained by their rarity on this staging area, where there is a striking male-bias (Jukema et al. 2001; Schmaltz et al. 2015). Birds were measured, weighed (±1 g), and banded with a metal band and an individual combination of four color bands and one leg flag. Sexing was based on wing length (Prater et al. 1977; Jukema and Piersma 2006). Age was determined on the basis of leg colour (orange for adults, greenish for first-year birds), the presence or absence of juvenile inner median coverts (Prater et al. 1977), and also by examining breeding plumage development (Meissner and Scebba 2005; Karlionova et al. 2008). For stable isotope analyses, we collected feather material for all birds captured and blood for 173 of them (163 males and 10 females), which were then used for stable isotope analysis.

To infer the winter origin of adult staging Ruffs, we measured $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ values in the vane of the 9th primary. For each bird, we clipped 2 cm of the inner vane at the feather's base using small scissors. Samples were then stored in paper envelopes until analysis. We did not consider young birds, as only a small proportion of them replace the outermost primaries in their first winter (Melter and Sauvage 1997). In parallel, we also retrieved samples of 9th primary (i.e. similar as above) of known origin: eight from Ruffs captured in November and December 2004 and 2005 in the Netherlands, and one from an adult female sold for consumption in the market of Mopti in Mali (14°29′45 N, 04°11′55 W) in February 2012.

After ringing, we took a blood sample (200 µL) by puncturing the wing vein and drawing blood into heparinized capillaries. Within 3 h after collection, blood samples were centrifuged for 12 min at 6900*g* in order to separate plasma and blood cells. Until freeze drying, plasma and blood cell samples were stored in glass vials in a freezer (−20 °C).

## Stable isotope analysis

Proximal feather vane material was washed in a 2:1 chloroform:methanol solution and dried.

For $\delta^{2}H_f$ analysis, dried washed feather material was weighed (*c.* 0.35 mg) into silver capsules and combusted under helium flow in a Hekatek furnace at 1350 °C interfaced with a Eurovector 3000 (Milan, Italy—www.eurovector.it) elemental analyser. The resultant $H_2$ gas was measured for $\delta^{2}H$ in an Isoprime (Crewe, UK) continuous flow stable isotope mass spectrometer and corrected for H exchange using the comparative equilibration technique of Wassenaar and Hobson (2003), using three keratin calibrated standards: Caribou Hoof Standard (CBS −197‰), Commercial Keratin Standard (SPK −121.6‰), and Kudu Horn Standard (KHS −54‰). All measurements are reported in δ-notation as the non-exchangeable feather H component in parts per thousand (‰) relative to the Vienna Standard Mean Ocean Water (VSMOW)—Standard Light Antarctic Precipitation (SLAP) scale. Based on replicate within-run measurements of standards ($n = 5$ per run) measurement error was estimated to be ±2‰.

For $\delta^{13}C$ and $\delta^{15}N$ analyses, between 0.5 and 1.0 mg of feather or dried blood material was weighed into tin capsules and combusted online using a Eurovector 3000 (Milan, Italy—www.eurovector.it) elemental analyser. The resulting $CO_2$ and $N_2$ was 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 $CO_2$ or $N_2$ reference gas. Stable nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$) isotope ratios were expressed in δ notation, as parts per thousand (‰) deviation from the primary standards, atmospheric AIR and Vienna Pee Dee Belemnite (VPDB). Using previously calibrated internal laboratory standards (powdered keratin BWB II: $\delta^{13}C = -20.0‰$, $\delta^{15}N = -14.1‰$ and gelatine: $\delta^{13}C = -13.6‰$, $\delta^{15}N = -4.7‰$) within run ($n = 5$) precision for $\delta^{15}N$ and $\delta^{13}C$ measurements was ±0.15‰.

## Assigning wintering origin

We lack sufficient ground-truth samples to enable assignment by means of a discriminant function analysis. Therefore, we chose to follow the approach of Yerkes et al. (2008) to establish feather isotopic thresholds (cut-off values) to delineate wintering biomes of staging Ruffs. Isotopic thresholds were set on the basis of: (1) the relative difference in the abundance of plants using C3 and C4 photosynthesis pathways in Europe versus sub-Saharan Africa which would respectively show $^{13}C$-depleted and enriched tissue values (mean C3 plant $\delta^{13}C$ −28‰, C4 plant $\delta^{13}C$ −13‰, Peterson and Fry 1987), (2) on the land-use practices with high nitrogen input (manure) increasing $\delta^{15}N$ values (Hebert and Wassenaar 2001, 2005; Yerkes et al. 2008) and (3) the negative gradient in $\delta^{2}H$ feather values existing from sub-Saharan West Africa towards northwestern Europe (Bowen et al. 2005). Samples of known moulting locations were then used to assess the relevance of our inferences.

First, we expected that European winterers would show lower $\delta^{13}$C feather values. Most of these Ruffs winter in open agricultural areas and replace their 9th primary in early September (Koopman 1986) while they forage on soil invertebrates and spilled grain found in meadows or arable lands (Castelijns 1994) with C3 crops. In contrast, feathers grown in sub-Saharan Africa should present higher $\delta^{13}$ C values. Sub-Saharan Ruffs moult primary 9 in early October for males to a month later for the delayed females (Pearson 1981; Koopman 1986; OAG Münster 1998). During this time, Ruffs rely on food based on a C4-dominated environment, invertebrates, but also grass seeds (e.g. *Echinocloa* sp., *Panicum* sp.) found along the edges of the floodplains, which are then at their highest levels after the rains of June and July (Tréca 1990, 1994; van der Kamp et al. 2002a). Eventually, we expect feather $\delta^{13}$C values to reflect a broad C3 and C4 isotopic range from −25.8 to −10.8‰ taking into account a diet feather discrimination factor of 2.16‰ (Caut et al. 2009); see also Werner et al. 2016). We considered the median threshold $\delta^{13}$C value of −18.3‰ to distinguish birds that fed on a C3 vs. C4 dominated diet at time of moult. The presence of cultivated maize (*Zea mays*, a C4 plant) in Europe and the presence of Rice (*Oryza* sp., a C3 plant) in sub-Saharan Africa should not confound our expectations. By the time Ruffs are replacing primary 9, neither the rice in West Africa nor the maize in Europe is harvested and available for Ruffs.

We assumed that $\delta^{15}$N feather values higher than 10‰ were indicative of birds feeding in agricultural areas subjected to high nitrogen input. In Europe, intensive agricultural practices are common with regular application of manure on lands. Animal waste, as an additional source of nitrogen, may have typically elevated $\delta^{15}$N values (from 10 to 25‰ compared to synthetic fertilizer (−4 to +7‰; Kendall 1998), which would be reflected in feather $\delta^{15}$N values (e.g. Hebert and Wassenaar 2001, 2005; Yerkes et al. 2008; Coulton et al. 2010). In comparison, land use in sub-Saharan Africa is extensive, but locally Ruffs may use pastures with high livestock densities. This would make it possible to encounter sub-Saharan individuals showing elevated feather $\delta^{15}$N values too.

Eventually, in the case of feathers showing low $\delta^{13}$C and low $\delta^{15}$N values, we assumed that birds used natural or extensive terrestrial or freshwater C3 habitats (i.e. not subjected to input of manure) while feeding on low trophic levels, as would indicate low $\delta^{15}$N values (Post 2002). Because Ruffs are associated primarily with freshwater (Cramp and Simmons 1983), this corresponds to natural freshwater wetland areas in Europe or perhaps ricefields. However, considering that rice cultivation is widespread in Africa and the high heterogeneity in farming practices there, we cannot rule out the use of non-harvested rice fields during the period of moult. To be conservative, we did not predict a specific geographical origin for these birds.

We thus ended up with four possible isotopic environments reflecting a bird's early wintering location and habitat (Fig. 2): (A) European agricultural areas with high N input with C3 foods (i.e. $\delta^{13}C < -18.3‰$, $\delta^{15}N > 10‰$), (B) Natural wetlands or rice fields in unknown wintering quarters ($\delta^{13}C < -18.3‰$, $\delta^{15}N < 10‰$), (C) sub-Saharan floodplains with C4 foods and areas grazed by livestock ($\delta^{13}C > -18.3‰$, $\delta^{15}N > 10‰$), (D) sub-Saharan floodplains ($\delta^{13}$ $C > -18.3‰$, $\delta^{15}N < 10‰$). We fully recognize that these cutoffs are somewhat arbitrary and that birds can be misplaced using these criteria. However, this approach was the most parsimonious given the data.

We chose to not set an isotopic threshold for $\delta^{2}$H and kept a more explorative approach. This is because Ruffs use freshwater wetlands and agricultural areas that are subjected to many processes and anthropogenic activities (e.g. intense evaporation, use of water of mixed origin, and ground waters) leading to large variation in the incorporation of deuterium into the diet and then in animal tissues (Bowen et al. 2005; Oppel et al. 2011; Hobson et al. 2012; Gutiérrez-Esposito et al. 2015). Nevertheless, at time of growth of primary 9, we expected to find a tendency for higher $\delta^{2}$H values in feathers grown in sub-Saharan than in feathers grown in Europe (Bowen et al. 2005). In sub-Saharan Africa, water feeding the floodplains of Senegal and Mali may importantly originate from May to July rains in the Guinean Highlands, which $\delta^{2}$H values range from −22 to 10‰ (SD ranged from 9.6 to 12.8‰) according to $\delta^{2}$H monthly mean values from the Global Network of Isotopes in Precipitation (GNIP) database administrated by the International Atomic Energy Association and World Meteorological Organization (IAEA/WMO 2001). In comparison, the $\delta^{2}$H values of September precipitation over Western Europe may range from −54 to −22‰ (SD ranged from 8 to 9.6‰). Finally, we examined the contribution of deuterium to the observed variation in feather $\delta^{13}$C and $\delta^{15}$N measurements with principal component analysis (PCA). This approach allowed us to incorporate our assignment criteria based on $\delta^{13}$C and $\delta^{15}$N feather values (i.e. isotopic environment A, B, C, D as supplementary qualitative variables), while taking into account the part of the overall observed variation explained by $\delta^{2}$H measures in feathers.

## Migration stages during northward migration

We isotopically characterized the staging grounds of Ruffs used 2 weeks to a month before their arrival on our study site in the Netherlands from $\delta^{13}$C and $\delta^{15}$N measures in

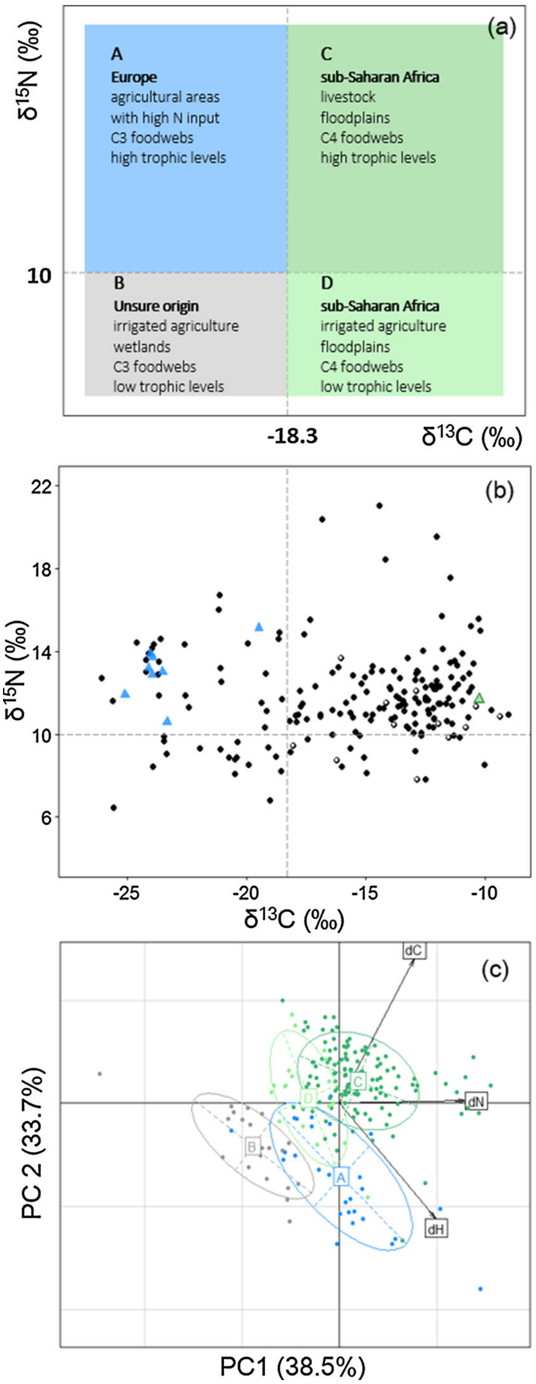

**Fig. 2** **a** Expected wintering isotopic environments for spring migrant Ruffs based on $\delta^{13}$C and $\delta^{15}$N thresholds values in feathers, **b** primary feathers $\delta^{13}$C and $\delta^{15}$N reflecting isotopes ratios of moulting areas in either sub-Saharan Africa or Europe. *Filled* and *open dots*, respectively, represent samples from males and females captured on the staging area in Friesland and for which wintering origin in unknown. *Filled triangles* (in *blue*) represent samples from males at known wintering site in the Netherlands, the *filled triangle* (in *green*) represent a sample from a female collected on the local food market in Mopti, Mali. **c** Principal Component (PC) biplot of $\delta^{13}$C, $\delta^{15}$N and $\delta^{2}$H measurements in feather samples from staging Ruffs collected in Friesland during spring migration 2012. Ellipses grouped 67% of the individuals assigned to each isotopic environment *A*, *B*, *C*, and *D* (see **a**)—here as qualitative supplementary variables). PC1 and PC2 refer to the principal component axes scores on the *first* and *second axes*, respectively. The amount of variation accounted for by the *axes* are shown in *brackets* (colour figure online)

plasma and blood cells. We assumed a half-life of 6 and 15 days for plasma and blood cells, respectively, as determined isotopically in a similarly sized shorebird, the red knot *Calidris canutus* for carbon stable isotopes (Klaassen et al. 2010). We considered that incorporation rate to be equivalent for the nitrogen stable isotope (van Gils and Ahmedou Salem 2015; but see Dietz et al. 2013). Then we used the isotopic thresholds set above for feathers, which we adjusted for blood cells and plasma tissues. For carbon, we considered an isotopic threshold value of $\delta^{13}C = -20.4$‰, which corresponds to the median value of C3 versus C4 dominated diet ($\delta^{13}C = -20.5$‰) adjusted with a carbon diet-whole blood discrimination factor of 0.09‰ ($\Delta^{13}C_{\text{diet-whole blood}} = -0.199 \times \delta^{13}C_{\text{diet}} - 3.986$‰, Caut et al. 2009). For nitrogen, we considered a threshold value of $\delta^{15}N = 8.4$‰, which is the nitrogen threshold value for feather ($\delta^{15}N = 10$‰) to which we subtracted the difference of 1.6‰ between nitrogen diet-feather and nitrogen diet-whole blood discrimination factors ($\Delta^{15}N_{\text{diet-whole blood}} = 2.25$‰, $\Delta^{15}N_{\text{diet-feather}} = 3.84$‰, Caut et al. 2009).

We kept the same rationale as in feathers but took into consideration that possible isotopic environments (Group A, B, C, D) reflected the birds' early spring locations and habitats. For isotopic environment B ($\delta^{13}C < -20.4$‰, $\delta^{15}N < 8.4$‰) this implies that the use of rice fields becomes more likely. In Africa, Ruffs can feed on spilled kernels in dry harvested ricefields (Tréca 1990), whereas in Europe Ruffs may benefit from the increasing tendency for post-harvest inundation of rice fields (Hortas and Masero 2012; Pernollet et al. 2015), which follows the first spring rains, and later the flooding of rice paddies for the new sowing (Wymenga 1999; Bacetti et al. 1998). After a change in isotope values of diet, tissues follow an exponential pattern of change (Karasov and Martinez del Rio 2007). We assumed a half-life of 6 and 15 days for plasma and blood cells, for carbon and nitrogen stable isotopes (see above). Ruffs, on average, stage 19–23 days in the Netherlands (Verkuil et al. 2010), which in principle allowed for enough time (i.e. three half-lives) for $\delta^{13}$C and $\delta^{15}$N plasma values of migrants to reach equilibrium levels on the local Dutch diet and for $\delta^{13}$C and $\delta^{15}$N blood cell values to nearly approach it.

Accordingly, we examined the $\delta^{13}$C and $\delta^{15}$N values of blood cells and plasma of Ruffs caught before 1 April.

Capture date should be close to true arrival date (Verkuil et al. 2010), and their blood isotope values likely reflect more of the isotopic environment of any previous staging area. From the $\delta^{13}$C and $\delta^{15}$N plasma values of these early birds, we verified the proposition that some of the Ruffs make a nonstop flight across the Sahara and Europe to the Netherlands (Jukema et al. 2001). Later, we considered Ruffs caught during the entire season to explore temporal patterns of changes in plasma and blood cell $\delta^{13}$C and $\delta^{15}$N values. We calculated log-linear time trends of changes in plasma and blood cell $\delta^{13}$C and $\delta^{15}$N values in Ruffs assigned to different wintering areas. Females were considered aside of males because of their delayed migration.

For birds wintering in European agricultural areas (A in Fig. 2a), we considered that plasma and blood cells isotopic values of these European Ruffs caught after 20 April were equivalent to the end points of $\delta^{13}$C and $\delta^{15}$N values in plasma and blood cells at equilibrium with the local diet.

All statistical tests involved were performed in R version 3.3.1 (R Development Core team 2016).

## Results

### Wintering origin

Values of $\delta^{13}$C and $\delta^{15}$N in feathers of known origin were in agreement with the independently set isotopic thresholds to discern wintering areas (Fig. 2). The $\delta^{13}$C and $\delta^{15}$N values in feathers grown in the Netherlands (mean ± SD, $\delta^{13}$C, −23.5 ± 1.68‰; $\delta^{15}$N, 13.0 ± 1.35‰), confirmed our expectation for an agricultural wintering habitat with high nitrogen inputs dominated by C3 foods (A: $\delta^{13}$C <−18.3‰, $\delta^{15}$N >10‰; see Fig. 2a, b). The isotope values of the one feather from a female wintering in Mali ($\delta^{13}$C = −10.3‰; $\delta^{15}$N = 11.7‰), were consistent with the suggestion that feathers grown in West Africa may present $\delta^{13}$C values close to those expected for a diet based on C4 plants, presumably an important food resource for Ruffs there.

Among the 199 Ruffs sampled during their stage in the Netherlands, 75% ($n = 149$) showed feather isotopic values consistent with those assumed to have a sub-Saharan wintering origin (Fig. 2a, c, d: $\delta^{13}$C > −18.3‰), of which 129 (given $\delta^{15}$N feather values >10‰—Fig. 2b) may have used areas grazed by cattle and/or feeding on higher trophic level foods. All females in the sample from Friesland were assigned to have wintered in sub-Saharan Africa (Fig. 2b). In contrast, only 29 male Ruffs (15%) seemed to have wintered in agricultural areas in Europe (A: $\delta^{13}$C <−18.3‰, $\delta^{15}$N >10‰; Fig. 2b), while 21 other males showed isotopic values with both depleted values in $^{13}$C and in $^{15}$N, probably indicating moulting areas in European freshwater wetlands or perhaps also rice fields, possibly in Africa (Chi square test: $\chi^2 = 169.3$, $df = 3$, $p < 0.001$). The $\delta^2$H values in feathers assigned to moulting areas in Europe (mean ± SD, −33.9 ± 24.4‰, $n = 29$) and in sub-Saharan (−40.6 ± 15.0‰, $n = 149$) both showed large variations and did not differ from each other (Mann–Whitney test, $W = 2619$, $P = 0.07$).

From the PCA analysis, the first two principal components (eigenvalues >1) accounted for 38 and 34% of the observed variations in feather isotopic values. The $\delta^{13}$C and $\delta^2$H feather values contributed the most (52 and 30%) to the first principal component (PC1), and were negatively correlated. Second principal component (PC2) was best described by $\delta^{15}$N (61%) and then by $\delta^2$H feather values (39%), in both cases positively.

### Spring staging grounds before arrival in Friesland

Among the 29 Ruffs assigned to European wintering quarters (group A), five birds were caught after the 20th of April, from which we took the average $\delta^{13}$C and $\delta^{15}$N values in blood cells and plasma to predict the expected endpoint values at equilibrium with the diet on the Dutch staging area as $\delta^{13}$C: −25.7 ± 1.2‰, $\delta^{15}$N: 12.3 ± 0.6‰ for blood cells and $\delta^{13}$C: −26.7 ± 0.9‰, $\delta^{15}$N: 12.2 ± 0.7‰ for plasma (mean ± SD, $n = 5$).

Out of all birds sampled, 55 Ruffs were captured before 1 April. Five were allocated to a European origin (9%), 42 (76%) to sub-Saharan Africa (38 in isotopic environment C, including one female and four in isotopic environment D), and eight birds (15%) could not be assigned to a particular location (isotopic environment B). The proportion of European wintering Ruffs in early catches did not differ from their proportion in overall catches [binomial test, $p = 0.26$, confidence interval (0.3–20%)].

Among the 41 early males that wintered in Africa, all had $\delta^{13}$C and $\delta^{15}$N plasma values indicating the use of agricultural areas in Europe as early as 2 weeks before arrival on the study site (Chi squared test, $\chi^2 = 123$, $df = 3$, $p < 0.001$) and so did the $\delta^{13}$C and $\delta\ ^{15}$N blood cells values for 30 of them, as early as a month before arrival in Friesland (73%, Chi squared test, $\chi^2 = 56.66$, $df = 3$, $p < 0.001$, Fig. 3). However, for 10 birds (24%), the $\delta^{13}$C and $\delta^{15}$N blood cell values were more contrasted to plasma values, falling in isotopic environment B and indicating the use of natural freshwater wetland areas in Europe or perhaps also rice fields (Fig. 3). A last male presented blood cell $\delta^{13}$C values indicative of C4 based diet (Group C), suggesting his presence in Africa in the month before his capture (Fig. 3). The single early arriving female showed plasma and blood cells isotope profiles indicative of agricultural habitat in Europe (Fig. 3a).

Over the entire passage period, all but one Ruff assigned to sub-Saharan Africa and of unknown wintering quarters (groups B, C, and D) presented $\delta^{13}$C and $\delta^{15}$N values in plasma already converged towards the local end point values for plasma (Fig. 4). This male caught on April 20 (plasma: $\delta^{13}$C −18.5‰ and $\delta^{15}$N 14.6‰, i.e. group C) provide the unique suggestion of a bird present in Africa within 2 weeks before his capture in the Netherlands.

The $\delta^{15}$N blood cell values of males assigned to Africa or unknown wintering quarters (groups B, C, and D), increased over time (Fig. 4, B: $F_{1\text{–}7} = 13.75$, $p = 0.02$, C & D: $F_{1\text{–}118} = 41.2$, $p \leq 0.001$) converging towards $\delta^{15}$N end point values expected in the Netherlands (Fig. 4) while it was not the case for blood cells $\delta^{13}$C values, all below the carbon threshold of −20.4‰ and even decreasing for sub-Saharan males (Fig. 4C, D: $F_{1\text{–}118} = 19.99$, $p < 0.001$). There was no such patterns for Ruffs that supposedly wintered in Europe (A: $F_{1\text{–}22} = 0.26$, $p = 0.61$). Finally, for all male Ruffs, we also observed a slight decrease in plasma $\delta^{13}$C values over the season, (Fig. 4A: $F_{1\text{–}22} = 6.91$, $p = 0.02$, B: $F_{1\text{–}7} = 4.66$, $p = 0.05$, C, D: $F_{1\text{–}118} = 23.95$, $p < 0.001$).

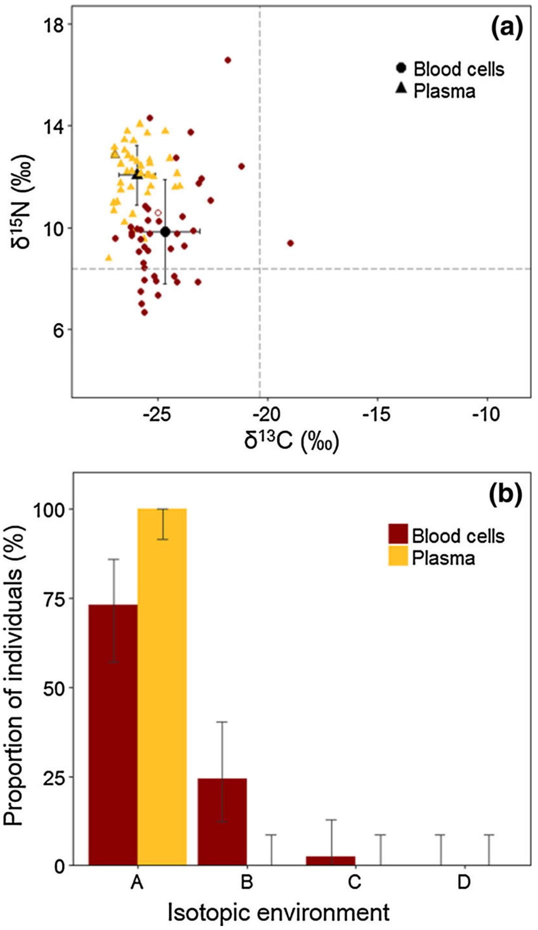

**Fig. 3** **a** Blood cells and plasma $\delta^{13}$C and $\delta^{15}$N (‰) of staging Ruffs caught before April 1st 2012 in southwest Friesland that were previously assigned to sub-Saharan wintering origin and mean values (*error bars* represent standard errors). These are all males, except for one female designated with *open symbols*. *Dashed lines* are indicative of isotopic thresholds for $\delta^{13}$C (−20.4‰) and $\delta^{15}$N (8.4‰) for blood cells and plasma tissues **b** Proportion of sub-Saharan Ruffs with blood cells and plasma isotopic signatures reflecting isotopic environments *A*, *B*, *C*, or *D* with 95% CI

As for females, two individuals captured on May 1 presented $\delta^{13}$C blood cells values indicative of C4 based diet (Fig. 4—$\delta^{13}C_{\text{blood cells}}$ of −18.3 and −19.6‰) and their presence in Africa in the last month. The eight other females had $\delta^{13}$C blood cells values already converged within expected values of the Netherlands.

## Discussion

Quantitative information on the migratory connectivity of endangered populations can establish the contemporary importance of critical sites or habitats, and prioritize management actions and conservation decisions. Our study shows that in 2012, the remnant staging population of Ruffs in the Netherlands consisted mainly of individuals wintering in sub-Saharan Africa, but nevertheless involved a considerable proportion of the small population of male Ruffs winterin g in Europe. For Ruffs wintering south of the Sahara, rice fields either in Africa or in the Mediterranean are likely to represent important fuelling areas. We found no evidence that Ruffs routinely make non-stop flights from West Africa to the staging site in the Netherlands. Instead, our results suggested that prior to their arrival in Friesland, Ruffs usually stopover in more southerly intensive agricultural areas.

The multi-isotopic values of winter-grown feathers from Ruffs staging in spring in the Netherlands showed differences between European and sub-Saharan wintering quarters although there was high variability (Fig. 2b, c). Intensive agricultural areas of Europe were satisfactorily delineated by the high $\delta^{15}$N threshold values characteristic of regular manure input on these lands. Above the $\delta^{13}$C threshold of −18.3‰, indicating a sub-Saharan origin, feathers showed high isotopic variability suggestive of the opportunistic feeding behaviour of Ruffs and/or the isotopic complexity of sub-Saharan freshwater wetlands (see Oppel et al. 2011). Our data indicate that during the growth of primary 9, sub-Saharan Ruffs occupied habitat dominated by C4 grasses, possibly at the interface between inundated zone and more xeric habitat given a relatively large range of feather $\delta^{13}$C values (e.g. Marra et al. 1998). The mostly enriched $\delta^{15}$N feather values may also reflect the influence of livestock. This could suggest wintering quarters in and near the Inner Niger Delta where livestock is an important resources and where Ruffs are abundant and could forage in floodplains habitats subject to grazing (Wymenga et al. 2002; Zwarts et al. 2009). Note that Ruffs

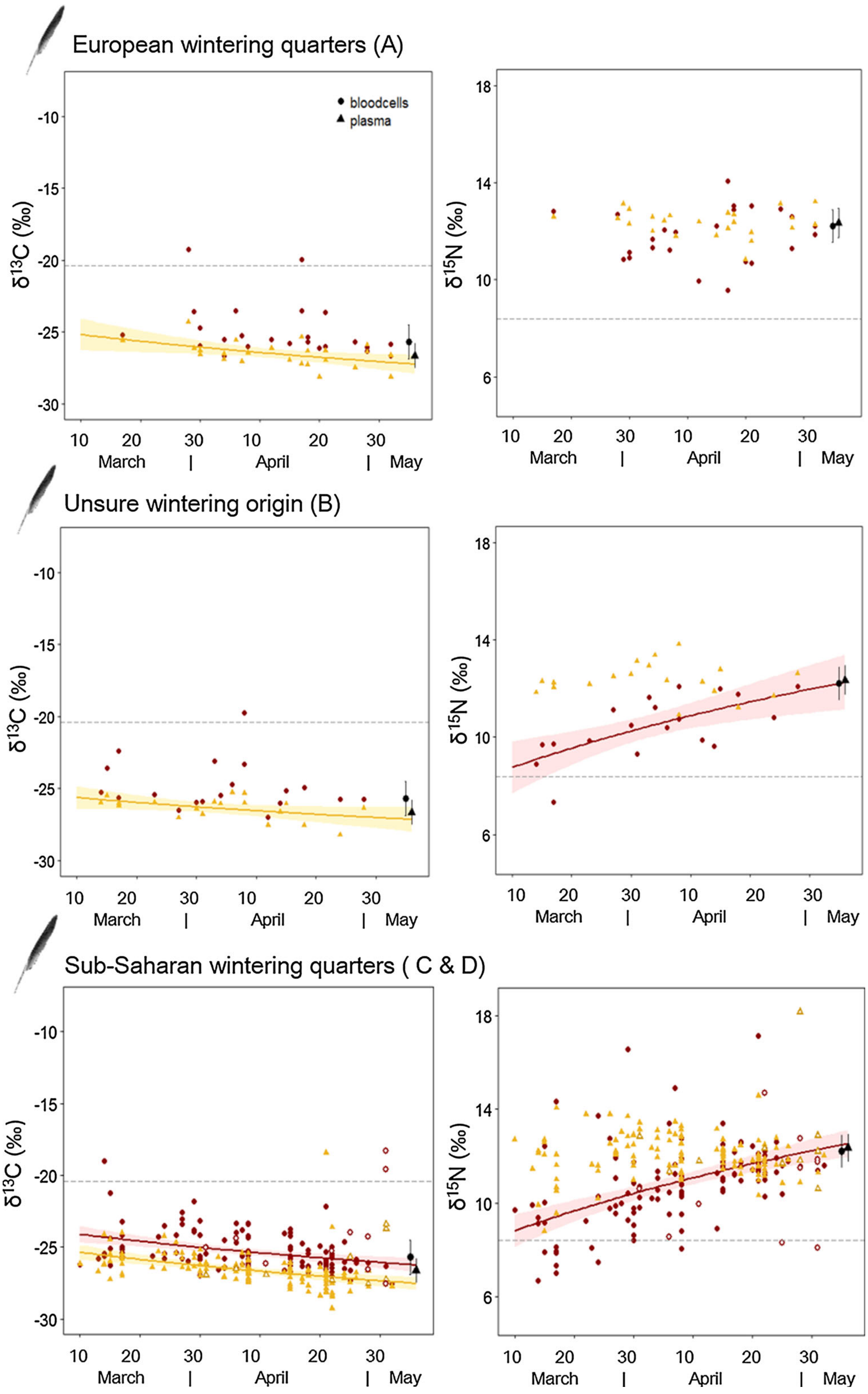

**Fig. 4** Temporal changes in the $\delta^{13}$C and $\delta^{15}$N values (‰) of blood cells (*dark red circles*) and plasma (in *yellow triangles*) of male ruffs in the course of the 2012 spring migration season in southwest Friesland. Only significant temporal trends are emphasized with a trend line and a 95% confidence contour. The $\delta^{13}$C and $\delta^{15}$N values (‰) of blood cells and plasma of females assigned to wintering quarters in sub-Saharan Africa, are represented by *open dark red dots* and open *yellow triangles,* respectively. *Dashed lines* are indicative of isotopic thresholds for $\delta^{13}$C (−20.4‰) and $\delta^{15}$N (8.4‰) for blood cells and plasma tissues (colour figure online)

have practically vanished from the Senegal River delta (Triplet et al. 2014). In contrast, the $\delta^{2}$H values of feathers presumably grown in Europe and those grown in sub-Saharan Africa did not differ.

Globally, our study provides new evidence that the assignment to wintering areas is challenging for ground-foraging species closely associated with freshwater wetlands, and notably tropical wetlands subjected to intense evaporation (Oppel et al. 2011; Hobson et al. 2012). Our study needs to be further augmented by more ground-thruth samples from Europe and sub-Saharan wintering quarters, and ideally combine multi-isotopic data with movement data from satellite tags. If isotopic values in tissues with crucial information on individual habitat use during the winter time could be linked, we could evaluate the feasibility of multi-isotopic large-scale geographical assignments for species strongly associated to wetlands (López-Calderón et al. 2017).

Despite these limitations, this study does provide important insights on the geographical aspects of the itineraries of Ruffs staging in spring in The Netherlands. The dairy farmland of the province of Friesland currently hosts mostly sub-Saharan wintering Ruffs. As the ca. 15% of the males staging in Friesland which had wintered in Europe is much more than the few percent expected on the basis of the ratio of wintering numbers (a few thousand in Europe; Gill et al. 1995; Qninba et al. 2006; Hortas and Masero 2012; Devos et al. 2012; Hornman et al. 2013) and hundreds of thousands in West Africa (Zwarts et al. 2009), Friesland appears especially important for these nearby wintering Ruffs.

Jukema et al. (2001) suggested that Ruffs wintering in Europe arrived earlier than sub-Saharan winterers on the staging site in Friesland. We found no evidence for this. Note, however, that Ruff numbers in spring 2012 represent less than 10% of the numbers observed by Jukema et al. (2001) in 1998 and, therefore, the whole context may be now very different. In particular, Jukema et al. (2001) suggested that European males arrived on the study site by late February, followed by the bigger flocks of African wintering Ruffs coming in by mid-March. Now, the first large flocks of Ruffs in Friesland are rarely observed before 10 March. It could thus be that the early passing European Ruffs are no more. Nevertheless, the Friesland staging area may remain a critical site for a perhaps currently restricted population of males wintering in northwest Europe.

In West Africa, the body mass of Ruffs at departure for spring migration should enable them to reach the Netherlands in a single non-stop flight (OAG Münster 1998). However, unlike red knots reaching the Wadden Sea (the Netherlands), their main stopover area in one non-stop flight of similar length from the Nearctic breeding grounds (Dietz et al. 2010), we found no indication of Ruffs landing on the Frisian staging site directly from sub-Saharan African origins. Only one bird, caught on 20 April, may have arrived non-stop from its sub-Saharan wintering quarters. Instead, $\delta^{13}$C and $\delta^{15}$N plasma values of Ruffs from West Africa suggest that Ruffs stopover in other intensive agricultural areas just before reaching Friesland (Figs. 3, 4). This is perhaps from elsewhere in the Netherlands or in neighbouring countries such as England and Belgium where Ruffs are also found during the winter. It will be important to identify those intermediate staging areas.

Indeed, all sub-Saharan wintering Ruffs newly arrived on the staging site (i.e. caught early) may have used intensive agricultural areas for 2 weeks, 73% for a month (Fig. 3). However, interestingly, the remaining birds showed more contrasting blood cells values, reflecting the use of habitats dominated by C3 terrestrial or freshwater aquatic primary production, within a month before they arrived in the Netherlands (Fig. 3a, b, group B). This either suggests previous intermediate stopovers in natural wetlands of Europe, where C3 plants dominate, or perhaps in rice fields of the Mediterranean or the Sahel. In the course of the migratory period, the global increase in $\delta^{15}$N values in blood cells of Sub-Saharan Ruffs while $\delta^{13}$C values remained low (Fig. 4) also suggests habitat switching from natural or man-made wetlands to intensive agricultural areas of Europe. During the pre-fattening period in Africa, Ruffs usually fuel on invertebrates along the receding water line of the floodplains (van der Kamp et al. 2002b; Zwarts et al. 2009), but there were also evidence of Ruffs fuelling on rice kernels. Tréca (1994) showed that from December onwards, the stomach of Ruffs caught in Senegal were filled almost exclusively with rice. In parallel, in Europe, January censuses in of southern provinces of Spain interestingly reported increasing numbers of Ruffs since the 1990s for Cataluña and since 2002 for Extremadura, highlighting the importance of rice fields for the species (Hortas and Masero 2012). Several resightings of Dutch colour-ringed birds in rice field areas in Spain, Portugal, and Italy in early spring (L.E. Schmaltz, unpublished data) eventually confirm the existence of early spring migratory connections between the Mediterranean and Dutch agricultural grasslands.

To conclude, despite the evident isotopic complexity of wetland habitats and habitats strongly modified by anthropogenic activities (Hebert and Wassenaar 2001; Oppel et al. 2011) and the opportunistic feeding behaviour of Ruffs, the use of isotopic threshold values helped us to obtain fair estimates of the non-breeding provenance and the use of spring staging habitats of the remnant population of Ruffs staging in Friesland in spring 2012. Most birds wintered in the sub-Saharan floodplains, while the dairy farmland of Friesland is a particularly important staging area for male Ruffs wintering in Europe. Sub-Saharan wintering Ruffs appear to use in sequence different habitats. Their spring migratory itineraries may comprise the use of ricefields in the Sahel, intermediate stopover(s) in ricefields and freshwater wetlands in Europe, before reaching intensive agricultural areas of Europe and only later the staging site in Friesland. Ruffs use heavily anthropogenic habitats during the non-breeding season, among which rice fields may be important during migration, which calls for new attention on the use of these habitats north and south of the Sahara, and on the importance of the threatened natural dynamics of the floodplains in the Sahel. The combination of more ground-thruted samples and movement data from satellite tags could tell in the future whether our insights are correct.

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