Discriminating geographic origins of migratory waders at stopover sites: insights from stable isotope analysis of toenails

Teresa Catry, Ricardo C. Martins and José P. Granadeiro

In this study we test the potential of stable isotope analysis to reveal wintering origins of waders mixing at stopover sites, using the dunlin Calidris alpina as a case study. We determined stable carbon (δ13C) and nitrogen (δ15N) isotope signatures of toenails of dunlins captured during winter at reference sites along the East-Atlantic Flyway, from Mauritania to the United Kingdom. Afterwards, during spring migration, dunlins were sampled at the Tagus estuary, Portugal, and assigned to their wintering grounds according to their stable isotope signatures. Toe nails from wintering dunlins at different sites had significantly different δ13C and δ15N signatures, despite some overlap in isotopic carbon ratios of birds from Morocco, Portugal and the UK. Among birds sampled during migration in Portugal, we found a clear bimodal pattern in δ13C values, corresponding to passage migrants from Mauritania (enriched δ13C values) and wintering birds from the Tagus estuary (depleted δ13C values). The first passage migrants from Mauritania appeared at the Tagus estuary by the end of March, with peak numbers during late April and early May. Our study provides evidence that isotopic signatures of toenails can play a deterministic role in tracing the wintering origins of migrant dunlins at their stopover areas. Toe nails, instead of feathers, can be the powerful and innovating tissue to sample in wader studies, allowing to bridge the gap in the field of migratory connectivity between sites used in different phases of the life cycle of waders.

Bird migration has always attracted scientific attention and the need to monitor seasonal movements of individuals and populations has promoted the continuous development of methods and techniques to investigate such behaviour (Wernham et al. 2002). Tracing the movement of birds allows a deeper understanding of connectivity among breeding, wintering and stopover sites throughout migratory flyways, which has important ecological, evolutionary and conservation implications (Webster et al. 2002). However, tracing the links among these areas is particularly difficult in species that show both broad geographic distribution and significant mixing among populations.

Waders perform some of the longest migratory flights amongst all birds and despite the relatively narrow latitudinal span in breeding distributions, most species show extensive wintering ranges and also use, along their migratory routes, numerous sites that act as refuelling stops – stopover areas. Here, populations of different wintering and breeding origins frequently overlap. In addition, several of these areas hold their own wintering populations that mix with passage populations of the same species during migratory periods (Delany et al. 2009). Among waders, geographically distinct populations or subspecies are often difficult to discriminate: morphological and plumage characteristics usually show extensive overlap, ringing recoveries provide few and often biased data, and genetic markers often lack discriminant power (Lopes et al. 2006). These circumstances prevent an accurate estimation and interpretation of several migration-related parameters, such as migratory schedules of different wader populations at their stopover areas.

Stable isotope analysis of bird tissues has been increasingly used to track movements of birds across isotopic gradients and thus assess their breeding or wintering origins (Hobson 1999, Atkinson et al. 2005). The choice of chemical elements and tissues is crucial to achieve the correct conclusions. Carbon and nitrogen have been mainly used to examine the relative contribution of terrestrial and marine-derived nutrients (Hobson 1999). Amongst birds, feathers and blood are the most commonly sampled tissues. Blood is a metabolically active tissue yielding an isotopic record that represents a short temporal window ranging from days to a few weeks from collection date (Hobson 1999). For this reason, blood is inappropriate to trace origins and migration patterns of animals (Bearhop et al. 2002). Feathers, being metabolically inert, show fixed signatures after synthesis (Hobson 1999) and thus provide geographical information of the moult ing grounds. However, for species such as waders, with unknown or complex moulting cycles (showing suspended or arrested moult), feather isotopic signatures are extremely difficult to interpret and can hardly provide accurate information on the geographical origins of sampled individuals.
Recently, stable isotope signatures of avian toenails were successfully used to infer diet, habitat use (Bearhop et al. 2003, 2004, Yohannes et al. 2010) and geographic origins (Clark et al. 2006) of different species. Toenails possess two important attributes not found in other avian tissues: they are metabolically inert but grow continuously and they integrate information on the diet of birds over a medium temporal scale prior to sampling, varying from weeks to few months (Bearhop et al. 2003). Therefore, toenails of migrants at stopover sites will invariably yield information from their pre-migratory grounds (Bearhop et al. 2003, Clark et al. 2006).

The dunlin Calidris alpina is a migratory wader with a circumpolar breeding distribution which winters along temperate and subtropical coastlines north of the equator (Delany et al. 2009). The Tagus estuary, Portugal, is one of the large European wetlands where wintering and passage migrant dunlins mix during migratory periods (Catry et al. 2011). Wintering dunlins include birds from the subspecies C. a. alpina (breeding mostly in northern Scandinavia) and C. a. schinzii (belonging to Baltic and probably to British and Irish breeding populations, Lopes et al. 2006, Delany et al. 2009). Spring migrants are thought to be mostly C. a. schinzii originating from Mauritania, and travelling north to their breeding grounds in Iceland.

In this study we investigate the power of stable isotope analysis (in toenails) to unveil the geographic origin of migratory dunlins at wetlands that act both as wintering and stopover sites. We also examine migratory schedules of dunlins at the Tagus estuary during spring migration in relation to phenological patterns described for the most important wintering wetland for waders within the East Atlantic Flyway, the Banc d’Arguin, Mauritania (Piersma et al. 1990).

### Material and methods

#### Sample collection

Dunlins were captured with mist-nets at Banc d’Arguin, Mauritania and Sidi Moussa, Morocco, during the winter (December–February), and at the Tagus estuary, Portugal, during both winter (as above) and spring periods (March–May; Table 1). We measured culmen length (nearest 0.05 mm), wing length (nearest 1 mm) and body mass (nearest 0.5 g). Fat was scored in a 0–8 scale according to Kaiser (1993) and muscle in a 0–3 scale following Barlein (1995).

Between 1 and 2 mm of nail were clipped from three to four toes of each bird using sharp scissors and stored in individual plastic bags. Toenails were also obtained from 13 dunlin specimens held at the Natural History Museum of London, collected during the winter at three British estuarine areas (Table 1). These samples were meant to represent the isotopic signature of dunlins wintering at the UK, to broaden the scope of our analysis within the East Atlantic Flyway.

#### Stable isotope analysis

Toenails were washed in double baths of 0.25 N sodium hydroxide solution alternated with baths of double distilled water to remove adherent contamination, and then dried at 50°C for 48 h. Between 0.30 and 0.40 mg of toenails were stored in tin cups for stable-carbon and nitrogen isotope assays. Isotopic ratios were determined by continuous-flow isotope-ratio mass spectrometry (CF-IRMS). Results are presented conventionally as δ values in parts per thousand (‰) relative to the Pee Dee Belemnite (PDB) for δ13C, and atmospheric nitrogen (N2) for δ15N.

#### Data analysis

In order to investigate differences in toenail isotopic signatures of dunlins sampled at the four wintering study sites, we used a linear discriminant analysis and evaluated the accuracy of predicted group memberships by cross-validation. Carbon and nitrogen isotopic values were log (x + 18) transformed prior to analysis to meet the assumptions of normality and homocedasticity. A second discriminant analysis was carried out excluding samples from the UK, in order to assign the origins of dunlins captured at Tagus during spring migration. Each sample was allocated to one of the wintering sites (Mauritania, Morocco and Tagus estuary) according to the highest probability of group membership. All samples that had a value of either canonical variable > 2.58 from any group mean were not classified, as we assumed them to have <1% chance of belonging to any particular group. The critical value of 2.58 represents the number of standard deviations away from the group mean above which group

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**Table 1. Location, sampling period, number of capture events and number (n) of dunlins sampled for stable isotope analysis.** Numerical digits after the month initials correspond to the first (1) or second (2) half of each month (e.g. Mar2 = second half of March). Samples from the UK are from museum specimens.

<table>
<thead>
<tr>
<th>Location</th>
<th>Sampling period</th>
<th>Capture events</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wintering</td>
<td>Banc d’Arguin, Mauritania</td>
<td>Dec 2009</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sidi Moussa, Morocco</td>
<td>Feb 2010</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rochford, Kilnsea and Emsworth estuaries, UK</td>
<td>Jan and Dec 1897</td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td>Tagus estuary, Portugal</td>
<td>Feb 2009</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dec 2009</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jan 2010</td>
<td>1</td>
</tr>
<tr>
<td>Migration</td>
<td>Tagus estuary, Portugal</td>
<td>Mar 2, 2010</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Apr 1, 2010</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Apr 2, 2010</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>May 1, 2010</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td></td>
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</table>
memberships is predicted to be < 1% (Atkinson et al. 2005). A discriminant analysis was also used to investigate the ability of body measurements (wing and culmen length) and physiological parameters (body mass, muscle, and fat scores) to classify dunlins during spring migration as wintering (local) or as passage migrants (from Mauritania).

A generalized linear model (GLM) with a binomial error distribution and a logit link function was used to investigate the probability of occurrence of passage migrant dunlins at the Tagus estuary as function of date.

All analyses were carried out with R ver. 2.11.1 (R Development Core Team).

Results

Isotopic signatures of wintering reference populations

Carbon isotopic ratios of dunlins from Mauritania were significantly different from those of Tagus, Morocco and the UK, which formed an uniform group (F 3,39 = 16.66, p < 0.001, n = 42, followed by post-hoc Tukey tests; Fig. 1). In contrast, all wintering sites showed statistically distinct δ15N signatures (F 3,39 = 54.11, p < 0.001, followed by post-hoc Tukey tests).

The discriminant analysis correctly classified all samples from Mauritania, but percentage of correct assignment was considerably lower within the other sites (55.6% for Morocco, 83.3% for Tagus estuary, 84.6% for UK). When excluding the samples from the UK, the discriminant analysis correctly predicted group membership in 90% of cases, showing 100% correct classification for Mauritania, 88.9% for Morocco and 83.3% for the Tagus estuary.

Assigning dunlins to their wintering grounds during spring migration

Overall, the 57 samples collected during the migration period (second half of March to early May) at the Tagus estuary did not fit well within the three reference sites (UK excluded), with approximately 40% of all individuals falling outside the 1% criteria of group membership. In addition, a high proportion of birds assigned to Morocco (50%, n = 10) and to the Tagus estuary (17%, n = 6) had low (<75%) classification probabilities. Indeed, δ13C and specially δ15N signatures from dunlins captured during spring migration show a larger variance compared with that of individuals sampled during the winter at reference sites (Fig. 1).

However, we did not find a significant correlation between δ15N values of local wintering dunlins during spring migration and their sampling date (Spearman correlation: r = 0.11, p = 0.608, n = 23).

There was a bimodal pattern in δ13C signatures: one group showed enriched δ13C values, more closely related with Mauritania signatures, and another group exhibited lower δ13C values, more similar to those from the Tagus estuary and Morocco (Fig. 1). For the purpose of the following analysis, birds with δ13C > −8‰ will be referred to as ‘passage migrants’, representing birds that spent the winter in Mauritania. Birds with δ13C < −12‰ will be considered to belong to the Tagus estuary wintering population.

Wintering and passage migrants revealed an almost complete overlap in body measurements (wing and culmen length) and physiological parameters (body mass, muscle, and fat scores; Table 2). In fact, a discriminant analysis using these variables was able to correctly classify only 66.1% of the birds in these two groups, and 16% of these had posterior probabilities < 60%

Table 2. Mean ± SD values of body measurements and physiological parameters of dunlins captured during spring migration at the Tagus estuary, classified as ‘wintering’ (n = 23) and ‘passage migrants’ (n = 34) according to their isotopic signature.

<table>
<thead>
<tr>
<th></th>
<th>Wintering</th>
<th>Passage migrants</th>
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<tbody>
<tr>
<td>Wing length (mm)</td>
<td>118.2 ± 4.0</td>
<td>112–125</td>
</tr>
<tr>
<td>Culmen length (mm)</td>
<td>31.4 ± 2.9</td>
<td>27.3–37.4</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>51.1 ± 7.3</td>
<td>39.0–67.4</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.0 ± 0.6</td>
<td>1–3</td>
</tr>
<tr>
<td>Fat</td>
<td>3.7 ± 2.3</td>
<td>1–7</td>
</tr>
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</table>
Migratory patterns of dunlins as revealed by stable isotopes

The first two passage migrants from Mauritania were captured at the Tagus estuary on the 24 and 31 of March, corresponding to 18% of the birds sampled during the second half of this month (n = 11). There was a significant increase in the proportion of passage migrants with date (z = 3.61, p < 0.001, n = 68, Fig. 2). According to the model, and assuming equal capture probability between wintering and passage migrants, 50% of the dunlin present at the Tagus estuary by the end of the first half of April originate from Mauritania, and represent > 80% of all birds during the last week of April and early May (Fig. 2).

Discussion

Despite being among the best studied avian groups, waders have seldom been used as model species for stable isotope studies, mostly for two reasons: first, wader moulting processes are poorly understood and highly variable (Prater et al. 1977) limiting the use of feathers to assign individual birds to geographic populations. Second, there are no clear isotopic gradients in wader habitats across large geographical scales, which prevent modelling isotope ratios as a function of latitude (Wunder et al. 2005). Our study shows, however, that stable isotopes help assigning dunlins to their wintering grounds, as long as a suitable tissue is selected for sampling. Although isotopic reference values of wintering dunlins may show seasonal and inter-annual variations, the magnitude of these variations is unlikely to mask the clear segregation in carbon signature among groups. These findings are likely to be applicable in many areas where wintering and passage birds co-occur during migration.

Tracing wintering origins of dunlins using stable isotope analysis

Isotopic signatures of dunlins from Mauritania are very distinct from those of European and Moroccan birds, these later presenting considerable overlap mainly in δ13C values. The more enriched δ13C values found in Banc d’Arguin, Mauritania, could partly be explained by the low freshwater influence (Hobson 1999) and by the large extent of seagrass beds (Conolly et al. 2005) compared with European estuaries. Nonetheless, given the magnitude of the differences found, also in comparison with samples from the saline environment of Sidi-Moussa, Morocco, it is more likely that the differences are due to distinct baseline values in the particulate organic matter of each site.

On the other hand, the significantly lower δ15N values found in dunlins from Mauritania could be due to differences in diet composition. Although prey consumed by dunlins is not considerably different among sites (Goss-Custard et al. 1977, Zwarts et al. 1990, Santos et al. 2005), dunlins at Banc d’Arguin eat remarkably smaller prey than elsewhere (Zwarts et al. 1990). Given that prey size is often positively correlated with δ15N signatures (Ménard et al. 2007), the consumption of smaller prey could lead to lower δ15N values. However, several other mechanisms, such as the degree of freshwater and/or terrestrial influence and the amount of anthropogenic inputs, may affect nitrogen isotopic ratios in intertidal areas (Hobson 1999, Ogden et al. 2005). Thus, the large differences found among some of the study sites suggest that the sources of nitrogen are very distinct and site-specific, as proposed in previous studies (Atkinson et al. 2005).

During spring migration the Tagus estuary acts as a stopover site for several thousand migrating dunlins that mix with the local wintering population (Catry et al. 2011). South of Tagus, there are only two important areas for wintering dunlins: Mauritania, holding the largest populations across West Africa (1 030 000 dunlins – Hagemeijer et al. 2004) and with much lower importance, Morocco (40 000 dunlins – Dakki et al. 2001). During spring migration at Tagus estuary two groups of individuals were clearly segregated by their δ13C values, strongly suggesting that dunlins with enriched δ13C signatures (> -8‰) belong to the wintering population of Mauritania. Most individuals with lower δ13C values (< -12‰) fall well within the range obtained for wintering Moroccan and Portuguese dunlins, but the existing evidence suggest that these birds mostly belong to the Tagus wintering population. Indeed, the large majority of these dunlins were captured before mid April, whilst numbers of wintering birds in Morocco only drop significantly in the second half of April (El Hamoumi and Dakki 2010), suggesting later departure dates.

Overall, the reduction in the proportion of birds with depleted vs enriched δ13C values fits well with bird counts carried out in the Tagus estuary. Count data reveal a peak of passage migrants the second half of April (Catry et al. 2011). In addition, telemetry studies have shown that part of the wintering dunlin population do not leave the estuary before late April, largely mixing with passage migrants (Catry et al. unpubl.).

Figure 2. Predicted probability of occurrence of passage migrant dunlins at the Tagus estuary fitted by a logistic regression (solid line). Histograms represent the distribution of capture events with migrant (top) and wintering (bottom) dunlins.
Nitrogen isotope ratios of dunlins sampled during spring migration were generally lower and showed larger variation than those found in wintering populations of both Mauritania and the Tagus estuary. The temporal mismatch in sample collection between the two periods (which varied from 1.5 to 4.5 months) might explain the differences found in $\delta^{15}N$ values. The period of information integration of stable isotopes in toenails varies according to species and nail growth rate. Deposition of keratin is a continuous process and thus the nail tip probably has a combination of old and new keratin, providing information over a medium temporal scale, from weeks to few months (Bearhop et al. 2003). As a consequence, samples collected during the winter might integrate information from the early winter, whilst samples collected in spring most probably integrate information from late winter. Important seasonal variations in the availability of benthic invertebrates are common and shorebirds are known to vary their diet accordingly (Zwarts et al. 2003). As a consequence, samples collected during the winter might integrate information from the early winter, whilst samples collected in spring most probably integrate information from late winter. Important seasonal variations in the availability of benthic invertebrates are common and shorebirds are known to vary their diet accordingly (Zwarts and Wanink 1993). However, the lack of a significant correlation between $\delta^{15}N$ values of local wintering dunlins during spring migration and their sampling date, meaning that the temporal mismatch could only partially explain the variation obtained.

Morphometrics, mainly bill length, have been used to trace dunlin migratory routes, linking breeding to wintering grounds (Pienkowski and Dick 1975, Hallgrímsson 2010). However, our data indicates that body measurements and physiological parameters alone are overall inappropriate to efficiently segregate dunlins by wintering origin.

**Migratory patterns of dunlins as revealed by stable isotopes**

The first migrant dunlin from Mauritania was recorded at the Tagus estuary on the 24 of March, but the bulk of migrants arrived during late April and early May. These schedules match with the departing period recorded by Piersma et al. (1990) in Mauritania (24 March–13 May; average date 29 April). The wide temporal window of migratory passage at the Tagus estuary also reinforces the hypothesis of intraspecific differences in departure timings of dunlins from their wintering grounds at Banc d’Arguin, which can be related to 1) sex differences in the optimal arrival time at the breeding grounds and 2) differences in breeding destinations (Piersma et al. 1990). Although dunlins were not sexed, bill length of early migrants covers a large range (26.0–36.75 mm), suggesting that both male and female dunlins are involved in early migratory movements. As for the differences in breeding destinations, although most dunlins wintering in Mauritania belong to the Icelandic C. a. schinzii breeding population (Pienkowski and Dick 1975, Delany et al. 2009), at least part of the C. a. schinzii populations breeding in Britain and Ireland and in the Baltic region also winter in West Africa, namely in Mauritania (Wernham et al. 2002, Thorup et al. 2009). The UK and Baltic populations, breeding at lower latitudes, might leave first their African wintering grounds in comparison with the Icelandic population, resulting in the observed bimodal pattern in timing of departure from Banc d’Arguin (Piersma et al. 1990) and also in the timing of stopover at the Tagus estuary.

**Acknowledgements** – We acknowledge all people that collected dunlin samples: Jutta Leyrer, Theunis Piersma (Mauritania), Ricardo Lopes, Hamid Idrissi and Latifa Joulami (Morocco). Hany Alonso, Miguel Lecoq, Ana Almeida, Inês Catry and Maria Dias helped with captures in Portugal. Mark Adams and Robert Prys-Jones, curators from the Natural History Museum of London, kindly provided samples from UK museum specimens. Paulo Catry provided helpful comments on the manuscript. This study was supported by FCT through Project PTDC/MAR/66319/2006 and grants to TC (SRFH/BPD/46967/2008) and RCM (SRFH/BD/44871/2008).

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