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Research article

Tissue- and diet-dependent stable carbon and nitrogen isotope discrimination: a calibration study in a captive shorebird species

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In ecology, stable-isotope ratios are widely used to determine diets of organisms and reconstruct food webs. This is usually done by analyzing the stable-isotope ratios of nitrogen ($\delta^{15}N$), which increase with increasing trophic level, and those of carbon ($\delta^{13}C$), which correlate with the $\delta^{13}C$ value of food source(s) and generally differ between terrestrial and marine food sources. Assimilation of food changes stable-isotope ratios, resulting in different values between the food source and its consumer. These differences are known as isotope trophic discrimination factors and, if known, can be used to determine from the stable-isotope ratios in the consumer's tissue what the consumer has been eating. What is often ignored is that discrimination factors can differ between consumer's food sources and also between tissue types. Therefore, we performed a controlled feeding study in red knots *Calidris canutus* to determine discrimination factors between different food sources and red knot tissues. We kept two groups of red knots in captivity on a stable diet, one group feeding on mudsnails and the other on Trouvit pellets, for several months, during which the birds molted their feathers. We analyzed $\delta^{13}C$ and $\delta^{15}N$ in both food sources and in five red knot tissues (blood cells, blood plasma and three feather types) and subsequently calculated the isotope discrimination factors. We confirmed that the discrimination factors differed between tissues, and also between diets. Our values deviated from general averages reported in reviews on a wide range of animals/birds, but were very similar to values from previous red knot and dunlin studies. We therefore think that our discrimination factors can be used in future stable isotope studies, not only on red knots, but also on other marine shorebird species and plea for careful consideration of using the right discrimination factors.

Keywords: $\delta^{13}C$, $\delta^{15}N$, discrimination factor, red knot, shorebird, stable isotope

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Introduction

Stable-isotope analyses are commonly used in ecology. One of the major applications lies in the reconstruction of a consumer's diet based on stable-isotope ratios in its tissues (Peterson and Fry 1987, Nielsen et al. 2018). The molecular building blocks that make up consumer tissues are derived from their food either directly or after some modifications and consumer stable isotopes ratios therefore reflect those of their food source one way or the other (Smith and Epstein 1970). The most-used isotope ratios in ecology are those of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$), expressed as $\delta^{13}C$ and $\delta^{15}N$ in ‰, relative to international standards (Mariotti 1983, Coplen 1994; see also Material and methods). The combination of $\delta^{13}C$ and $\delta^{15}N$ is particularly powerful as it helps to reconstruct food webs (Wada et al. 1991, Layman et al. 2012). Namely, variation in $\delta^{13}C$ mainly results from differences in the photosynthetic pathways at the base of the food chain (Smith and Epstein 1971, Deines 1980), whereas $\delta^{15}N$ is related to the trophic level, as it generally increases with the assimilation of food (DeNiro and Epstein 1981, Minagawa and Wada 1984). This so-called enrichment of ^{15}N causes a difference in $\delta\ ^{15}N$ between the diet and its consumer tissues, known as the trophic discrimination factor, $\Delta^{15}N$ (Bond and Hobson 2012). Discrimination with trophic level also occurs for ^{13}C ($\Delta^{13}C$; DeNiro and Epstein 1978), but whereas $\Delta^{15}N$ is mostly positive, $\delta^{13}C$ values can both increase or decrease from lower to higher trophic levels (Tieszen et al. 1983, Post 2002, Caut et al. 2009).

To estimate relative diet compositions of consumers from stable-isotope analyses, it is essential to use correct discrimination factors (McCutchan et al. 2003, Bond and Diamond 2011, Phillips et al. 2014). Feeding experiments with a known isotopic composition of the food have shown that discrimination factors can vary substantially between consumer species (Vander Zanden and Rasmussen 2001, Caut et al. 2009) and between consumer tissue types (DeNiro and Epstein 1978, DeNiro and Epstein 1981). However, many studies use averages of these experiments (both of consumers and tissue types, e.g. Post 2002), which might lead to incorrect diet estimations and conclusions (McCutchan et al. 2003, Bond and Diamond 2011). Healy et al. (2017) created the R package (www.r-project.org) SIDER, which estimates trophic discrimination factors for certain species, based on their ecology and phylogenetic relatedness. However, Stephens et al. (2022) showed some limitations of SIDER estimating $\Delta^{13}C$ in herbivores and omnivores, as those values are affected by diet source (more than phylogenetic relatedness). Although SIDER potentially provides accurate estimates for trophic discrimination factors, controlled feeding experiments provide the best discrimination factor estimates (Healy et al. 2017, Stephens et al. 2022). It is therefore important to carry out feeding experiments across taxonomic groups of consumers and calculate discrimination factors for often used tissues (Gannes et al. 1997, Martínez Del Rio et al. 2009).

In birds, blood and feathers are the easiest tissues to sample, non-invasively, and their bulk stable isotope ratios contain valuable information. Besides diet composition, stable isotopes from those tissues are for example used to determine breeding or non-breeding grounds, migration routes, and timing of migration (Rubenstein and Hobson 2004, Dietz et al. 2010, Cherel et al. 2016, Cruz-Flores et al. 2018). Especially the combination of the study of stable isotopes in blood and feathers is a powerful tool to study migration, as they integrate dietary information across different time scales (Hobson 1999). Specifically, blood contains information about the recent diet and feathers, which are metabolically inactive, contain dietary information from the moment and location they were grown (Mizutani et al. 1990, Hobson 1999). Stable-isotope values within blood plasma are transferred from diet values in about two weeks, while in blood cells this takes about two months (Hobson and Clark 1993, Klaassen et al. 2010, Lourenço et al. 2015). With known turnover rates, blood plasma and blood cells can be used, for example, to calculate timing of migration, when isotopic ratios of their food sources differ between locations (Dietz et al. 2010, Oppel and Powell 2010). Stable isotopes in feathers can provide additional information on breeding or non-breeding locations, when their molting patterns are known (Chamberlain et al. 2000, Pain et al. 2004). Different feather types can be molted at different times of the year and can thereby contain information from multiple timepoints and locations within an individual (Payne 1972, Atkinson et al. 2005). So, for example, by sampling both blood and specific feather types just after their migration, both their pre-migration location and timing can be estimated (Chamberlain et al. 2000, Atkinson et al. 2005). Another tissue, less frequently used, are toe nails of birds, which grow continuously but have a lower turnover rate than blood (stable-isotope values in toe nails are in equilibrium with diet values in about four months) and therefore potentially provide additional information (Bearhop et al. 2003, Lourenço et al. 2015).

In shorebirds, a well-studied group containing mostly migratory species, stable isotopes are frequently used to study their ecology, such as their diet composition, habitat use and their position in food webs (Catry et al. 2016, Lei et al. 2021, Ersoy et al. 2022). However, experiments to determine discrimination factors have only been done with blood samples in two species, dunlin *Calidris alpina* and red knot *Calidris canutus*, and analysis of feather samples is lacking in those experiments (Supporting information; Ogden et al. 2004, Lourenço et al. 2015, van Gils and Ahmedou Salem 2015). Here, we present a calibration study in captive red knots, that were kept on a stable diet for several months, during which the birds molted. Newly grown feathers and blood (plasma and blood cells) were analyzed for $\delta^{13}C$ and $\delta^{15}N$. As diet composition might be an additional source of variation in trophic discrimination factors (Hilderbrand et al. 1996), we used two isotopically distinct diets. We report discrimination factors per tissue for future research, not only for red knots, but potentially also for other birds with similar ecologies.

Material and methods

Red knots *C. canutus* (Linnaeus, 1758) are migratory shorebirds, consisting out of six subspecies, that breed on the high-Arctic tundra and inhabit seashores around the globe during the non-breeding season (Buehler and Piersma 2008). The Wadden Sea is an important non-breeding area for the *islandica*-subspecies (Buehler and Piersma 2008). There, on the Dutch island of Griend, 20 adults were caught with mist nets in the night of 28 October 2019 (Table 1, timeline of experiment). The next day, they were moved to two outdoor aviaries (2 groups of 10 individuals each) in the Shorebird Facility of NIOZ Royal Netherlands Inst. for Sea Research (Vézina et al. 2006). In the first two months the birds were fed Trouvit pellets (consisting mostly out of soy and fishmeal; Trouw, Fontaine-les-Vervins, France) ad libitum and both fresh and seawater was always available. From 8 January 2020 onwards, one group received a diet of small marine mudsnails *Peringia ulvae* (Pennant, 1777) which is part of red knots' diets in the wild (Dekinga and Piersma 1993), while the other group stayed on the Trouvit diet, during the entire experiment. The first month, *Peringia* was taken from a frozen batch at NIOZ, that was collected in the Dutch Wadden Sea. On 7 February 2020, new *Peringia* were fished (by dragging a net over the sea floor) from a single location in the Dutch Wadden Sea, ensuring relatively constant stable isotope values, and stored in a −20°C freezer. This batch was fed from 9 February 2020 onwards (Table 1).

Food was sampled and stored in a −20°C freezer until stable isotope analysis. To check if the values were constant over time, we sampled weekly from 21 January to 13 May 2020, and afterwards at a few random moments (Table 1). Blood samples were taken at five moments (25 February, 16 March, 20 April, 11 August and 22 Septemper 2020). To do so, we pinched the brachial vein under the wing with a small needle and collected blood (60–120 µl) with a capillary glass tube, which was emptied in a 0.5 ml microcentrifuge tube. These samples were centrifuged for several minutes until the heavier blood cells were clearly separated from the blood plasma. Blood plasma was subsequently pipetted to another tube and both samples were stored in a −20°C freezer. The red knots molted from their grey and white non-breeding plumage into their red and black breeding plumage from mid-January to mid-May (with the peak of molt in April, Table 1). The newly grown body feathers were collected on 9 June 2020, by cutting the entire feather, two from both breast and back. Primary coverts ('standard' sixth primary covert, Atkinson et al. 2005) were collected on 6 October 2020, after they have been grown from mid-July to mid-September, by cutting the top half (~2 cm). Feather samples were conserved in small paper envelopes.

To prepare for isotope analyses, food and blood samples were freeze dried. *Peringia* samples required extra preparation, flesh was separated from the shell first, using tweezers under a microscope. However, large variations in $\delta^{13}C$ during test measurements indicated that the samples still contained carbonates, while $\delta^{15}N$ values were not affected. *Peringia* samples were therefore decalcified offline using hydrochloric acid, before measuring $\delta^{13}C$. This meant that each freeze-dried sample was weighed into exetainers and acid was added until bubbling stopped, after adding an additional amount of acid the exetainers were shaken overnight to dissolve all carbonates present. Excess acid was removed by washing with double distilled water after which the samples were freeze dried again. Feather samples were cleaned, to remove wax and possible dirt, by stirring them in a cup of ethanol and hexane subsequently, after which they were air dried. The tips of the feathers were used for the analyses.

All samples were weighted precisely into tin capsules (0.5–1.0 mg), folded and their stable isotope compositions ($\delta^{13}C$ and $\delta^{15}N$) were measured using a Flash 2000 EA with a MAS 200 autosampler that was connected via a CONFLO IV to DELTA V ADVANTAGE irMS (Thermo Scientific, Bremen, Germany). $\delta^{13}C$ and $\delta^{15}N$ of the samples were determined using certified standards throughout every individual analytical sequence. For $\delta^{15}N$ and $\delta^{13}C$ analysis Acetanilide, Casein and Urea were used. For $\delta^{13}C$-only analysis after decalcification Benzoic acid, Acetanilide and Urea were used. All values are expressed in δ (‰) notation relative to the Vienna PeeDee Belemnite (VPDB, $\delta^{13}C$), and Air ($\delta^{15}N$).

The isotope values of the *Peringia* samples differed between the first and second batch, and therefore only the values of

Table 1. Timeline of the experiment. Point events are indicated with an X and periods of molt are shaded. Light shading in 'Molt to breeding plumage' indicates a maximum molt score of 1 (1–5 growing breast feathers) throughout all individuals, darker shading is used when the molt score of any individual is higher than 1 (meaning > 5 growing breast feathers). Shading in 'Molt of primary coverts' indicates when the new 6th primary in any individual is growing (which grows simultaneous with the sampled 6th primary covert).

Month (2019 & 2020)	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
Capturing red knots	X											
Diet of 'Trouvit-group'	Trouvit -->											
Diet of '*Peringia*-group'	Trouvit >		*Peringia* (old)>	*Peringia* (new) -->								
Fishing *Peringia*				X								
Sampling food			X X X X X X X X X X X X X X X X X X					X	X	X	X X X	
Sampling blood				X	X	X				X	X	
Molt to breeding plumage												
Sampling breeding plumage								X				
Molt of primary coverts												
Sampling primary coverts												X

the second batch were used for the analysis (because these *Peringia* were fed from 9 February throughout the experiment, Table 1). Blood samples from the birds on the *Peringia* diet, taken in February and March were excluded from the analysis, because the switch to their stable diet (the second *Peringia* batch) was too recent and their values were still influenced by their previous diet on Trouvit and by *Peringia* values from the first batch.

Discrimination factors were calculated by subtracting the mean $\delta^{13}C$ and $\delta^{15}N$ of the food sources (means were used after showing the absence of trends over time, Supporting information) from the isotope ratios of the tissue samples. Including individual bird as random effect in the linear model that explained variation in discrimination factors, including the interaction of food and tissue type, did not improve this model (AIC comparison, Supporting information). The samples were therefore judged as independent. Differences in discrimination factors between tissues and food types were tested with ANOVA (data visually meet model assumptions), including the interaction between both, and subsequently differences between combinations of food and tissue type were analyzed using a Tukey's test. These analyses were performed in R (www.r-project.org, functions 'aov' and 'TukeyHSD').

Results

The $\delta^{13}C$ and $\delta^{15}N$ values in both food types did not follow a tendency over time (Supporting information), and were on average ± SD: $\delta^{13}C$: −23.98 ± 0.34‰ and $\delta^{15}N$: 3.56 ± 0.40‰ for Trouvit and $\delta^{13}C$: −21.33 ± 0.19‰ and $\delta^{15}N$: 11.69 ± 0.14‰ for *Peringia* (Fig. 1, Supporting information). The mean $\delta^{13}C$ and $\delta^{15}N$ values within the bird tissues per diet are specified in the Supporting information. Most discrimination factors were positive and fell in between −0.1 and 3.8‰ for $\Delta^{13}C$ and between 1.9 and 4.1‰ for $\Delta^{15}N$ (Fig. 2).

For different tissue types, discrimination factors differed, with generally lower values in blood than in the feathers (Fig. 2, Table 2). Specifically, for $\Delta^{13}C$ and in both diet types, the lowest values were found in blood plasma, followed by blood cells, and subsequently feathers (Fig. 2, Table 2). Amongst feathers, back feathers had lower $\Delta^{13}C$ values than breast feathers and primary coverts. For $\Delta^{15}N$, differences in discrimination factors were smaller than for $\Delta^{13}C$, and in both diet types, blood cells presented lower values than blood plasma and feathers (Fig. 2, Table 2). On the *Peringia* diet, $\Delta^{15}N$ values in blood plasma were statistically higher than in feathers, except for primary coverts which did not differ significantly. Contrastingly, on the Trouvit diet, $\Delta^{15}N$ values in blood plasma were lower than in feathers, although this difference was only significant with primary coverts.

Discrimination factors in tissues of red knots on a diet of *Peringia* were higher than on Trouvit, for both isotope ratios (range for $\Delta^{13}C$: 0.28–0.97‰ and for $\Delta^{15}N$: −0.07 to 0.47‰, Table 2), except for $\Delta^{15}N$ in all feather types, where discrimination factors did not differ between both diet types.

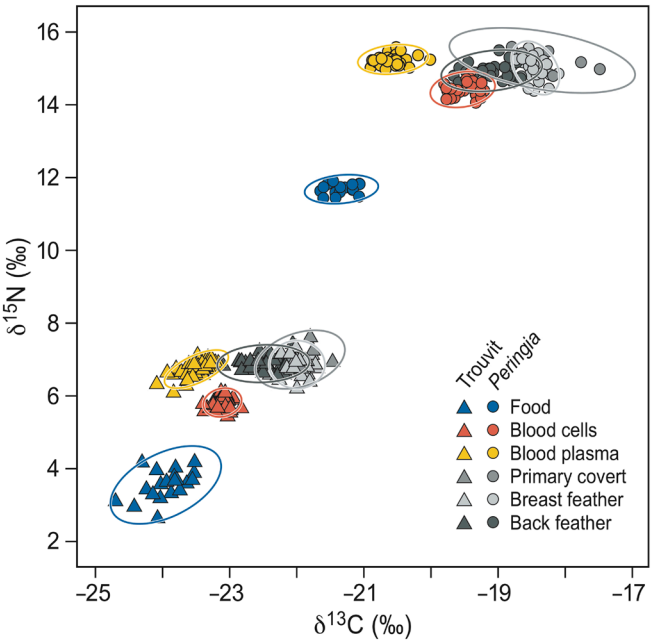

Figure 1. Stable isotope values of $\delta^{13}C$ and $\delta^{15}N$ of several tissues of red knots and their corresponding food sources. Points show all measured values and 95% confidence ellipses are created with the 'stat_ellipse' function in R (www.r-project.org, Fox and Weisberg 2011).

Discussion

Discrimination factors of $\delta^{13}C$ and $\delta^{15}N$ differed between tissues of red knots and also the type of diet affected the discrimination factors (Fig. 1, 2, Table 2).

Most experiments that report discrimination factors show differences between tissues (reviewed by Caut et al. 2009). However, differences between feather types as we found here, have not been observed often. Michalik et al. (2010) discovered that the presence of ^{13}C-depleted melanin lowers the $\delta^{13}C$ value in black feathers, and within feathers that contain both black and white, black parts also showed lower $\delta^{13}C$ values. This explained the results of Becker et al. (2007), who found lower $\delta^{13}C$ values in the black primaries of common murres than in their white breast feathers, which were molted while being kept on a constant diet. This mechanism, where the presence of melanin lowers the bulk ^{13}C values of dark feathers, could also explain the relatively low $\Delta^{13}C$ we found in red knot back feathers, which are mostly dark black in the breeding plumage, compared to the lighter black primary coverts and red breast feathers. In line with both abovementioned studies, $\Delta^{15}N$ did not differ between feather types (except for back feathers differing from primary coverts on *Peringia* diet).

For both isotope ratios, discrimination factors were bigger with the *Peringia* diet than with the Trouvit diet, in all tissues except for $\Delta^{15}N$ in feathers. This difference in discrimination factors between diets probably results from a different food quality. Trouvit is high-quality food, as it is protein-rich and produced for feeding fish, whereas *Peringia* are complete animals, collected from the wild, that consist of a wide array of

natural compounds. Multiple studies support the idea that $\Delta^{15}N$ decreases with increasing protein quality (Adams and Sterner 2000, Robbins et al. 2005, Perga and Grey 2010). This is in line with our results, assuming higher protein quality in Trouvit compared to *Peringia*, resulting in lower $\Delta^{15}N$ values in blood cells and plasma when feeding on Trouvit. This pattern of decreasing discrimination factors with higher dietary protein is less apparent in $\Delta^{13}C$ in the literature, but Stephens et al. (2022) show this relation in consumers on single-source diets and argue that this relation might have been overlooked in the past, because of the use of mixed-diets in experiments. In this study, where we used single-source diets, the results support what was found by Stephens et al. (2022), with lower $\Delta^{13}C$ values in Trouvit than in *Peringia*.

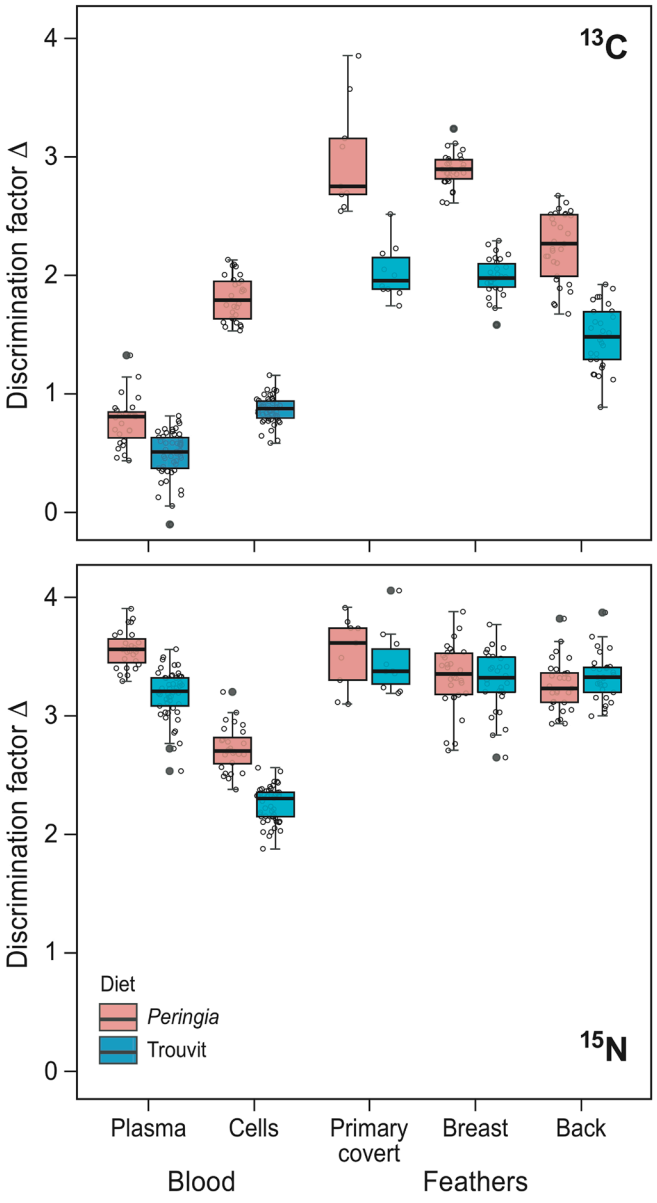

Figure 2. Discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) of several tissues of red knots, in captivity, which received a different type of diet.

The effect of diet on trophic discrimination factors becomes an issue when the goal of an isotopic sampling study is to reveal differences in diet composition. However, two very different food sources were used here, and the composition of Trouvit likely does not represent any potential food source that would occur in the wild. For future studies on shorebirds using stable isotopes, we therefore propose to only use the discrimination factors described here for *Peringia* (Table 2). Although Trouvit might be out of the range to be expected in the wild for food or prey organic matter composition, our results highlight the importance of considering that the composition of food likely affects discrimination factors.

To compare the discrimination factors we found (on *Peringia* diet) with published values, we first look into shorebirds. Shorebirds are a taxonomic group with usually a marine carnivore diet and discrimination factors are therefore likely to be similar between species. Within shorebirds, experimentally obtained discrimination factors have only been reported for red blood cells and plasma from dunlins and red knots (Supporting information; Ogden et al. 2004, Haramis et al. 2007, Klaassen et al. 2010, Lourenço et al. 2015). Values from our study are very similar to those studies (our $\Delta^{13}C$ and $\Delta^{15}N$ values for blood cells and blood plasma differ less than 0.5‰ from the averages of those studies), and are therefore likely suitable to be used in stable isotope studies of other shorebird species. Besides blood and plasma, Ogden et al. (2004) also reported discrimination factors for feathers of three individual dunlins, but it is unclear how these were calculated as they mention that these birds molted during a diet switch. This probably caused that two of the individuals show discrimination factors for both isotopes ($\Delta < -2$‰) that are far out of the usual observed range. However, the discrimination factors of the third individual ($\Delta^{13}C$ +2.2‰ and $\Delta^{15}N$ +3.4‰) fit within the range reported here.

When comparing our discrimination factors to general averages that are often used for stable isotope analyses (Post 2002 and Caut et al. 2009, both cited >1000 times), we

Table 2. Discrimination factors $\Delta^{13}C$ and $\Delta^{15}N$ of several red knot tissues on two diet types. Means (±SD) of discrimination factors are given. Letters indicate significant differences (ANOVA) in discrimination factors among tissues, within isotope and food type. p-values of t-tests, to test differences in discrimination factors between diets, within isotope and tissue, are shown. Discrimination factors on the *Peringia* diet, the shaded column, are recommended to be used in future research.

		Discrimination factor value		
	Tissue	*Peringia* diet	Trouvit diet	p-value
$\Delta^{13}C$	Blood cells	1.81 ± 0.19^{a}	0.87 ± 0.11^{a}	< 0.01
	Blood plasma	0.77 ± 0.20^{b}	0.49 ± 0.19^{b}	< 0.01
	Back feather	2.24 ± 0.29^{c}	1.48 ± 0.26^{c}	< 0.01
	Breast feather	2.89 ± 0.14^{d}	1.98 ± 0.16^{d}	< 0.01
	Primary covert	2.99 ± 0.46^{d}	2.02 ± 0.23^{d}	< 0.01
$\Delta^{15}N$	Blood cells	2.72 ± 0.19^{a}	2.25 ± 0.15^{a}	< 0.01
	Blood plasma	3.56 ± 0.16^{b}	3.18 ± 0.20^{b}	< 0.01
	Back feather	3.25 ± 0.22^{c}	3.32 ± 0.20^{bc}	0.95
	Breast feather	3.33 ± 0.28^{cd}	3.30 ± 0.26^{bc}	1.00
	Primary covert	3.53 ± 0.30^{bd}	3.45 ± 0.27^{c}	1.00

observe some differences. The trophic discrimination factors for nitrogen $\Delta^{15}N$ found here (ranging from +2.25 to +3.56‰, Table 2), fit within the widely used value of +3.4 ± 1‰ (average ± SD), as calculated by Post (2002), who averaged discrimination factors from various studies on different species. However, the average given by Post (2002) for carbon $\Delta^{13}C$, equal to +0.4 ± 1.3‰, is relatively low compared to our study (ranging from +0.49 to +2.99‰, Table 2). This deviation from the general average, shows the importance of species- or clade- and tissue-specific discrimination factors in stable-isotope studies, since the estimation of diet composition will be more accurate when using the relevant discrimination factors. When comparing our values (on *Peringia* diet) to those given for birds in Caut et al. (2009), frequently used as a source for discrimination factors of birds, we also find some differences. The measured $\Delta^{13}C$ and $\Delta^{15}N$ values we found are generally higher than those reported by Caut et al. (2009), with the biggest difference in $\Delta^{13}C$ in blood cells (+1.55‰), except for $\Delta^{15}N$ in feathers which is slightly lower in the study presented here (up to −0.51‰ in primary coverts). These differences probably originate from the large variation within the clade of birds (Caut et al. 2009, Supporting information), ranging for example from relatively small insectivorous warblers to larger piscivorous penguins and to herbivorous quails. Although the data is too limited to test if variation in discrimination factors within families is lower than between families, it is likely that species that are closely related, are more similar and therefore also have similar discrimination factors. For example, for a stable-isotope study on a certain warbler species, it would therefore probably be better to use known values of other warbler species rather than a general average of birds, which is obtained from other families.

For future research using stable isotopes, for example to reveal diet compositions of red knots or other marine shorebird species, we recommend using our tissue-specific discrimination factors on *Peringia* (Table 2), if species-specific discrimination factors are not available from scientific literature.

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Author contributions

Tim Oortwijn: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Visualization (equal); Writing – original draft (equal). **Luc de Monte**: Investigation (equal); Methodology (equal); Resources (equal). **Daniel P. Varley**: Data curation (equal); Formal analysis (equal); Visualization (equal). **Marcel T. J. van der Meer**: Resources (equal); Supervision (equal); Writing – review and editing (equal). **Jan A. van Gils**: Conceptualization (equal); Funding acquisition (equal); Supervision (equal); Writing – review and editing (equal).

Transparent peer review

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Data availability statement

Data are available in the Royal Netherlands Institute for Sea Research (NIOZ) data repository: https://doi.org/10.25850/nioz/7b.b.zd.

Supporting information

The Supporting information associated with this article is available with the online version.

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