METHODS

Using stable isotope ($\delta^{13}C$, $\delta^{15}N$) values from feces and breath to infer shorebird diets

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Abstract
The use of stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) from feces and breath offers potential as non-destructive tools to assess diets and nutrition. How stable isotope values derived from breath and feces compare with those from commonly used tissues, such as blood fractions and liver, remains uncertain, including understanding the metabolic routing of dietary nutrients. Here, we measured $\delta^{13}C$ and $\delta^{15}N$ from feces and $\delta^{13}C$ of breath from captive Red-necked Stints (Calidris ruficollis) and 26 species of wild-caught migratory shorebirds ($n=259$ individuals) and compared them against isotopic values from blood and feathers. For captive birds fed either cereal- or fish-based diets, differences in $\delta^{13}C$ between feces and lipid-free diet were small, $-0.2 \pm 0.5\%e$ and $0.1 \pm 0.3\%e$, respectively, and differences in $\delta^{15}N$, $-0.7 \pm 0.5\%e$ and $-0.5 \pm 0.5\%e$, respectively. Hence, $\delta^{13}C$ and $\delta^{15}N$ values from feces can serve as proxies for ingested proteinaceous tissues and non-soluble carbohydrates because isotopic discrimination can be considered negligible. Stable isotope values in plasma and feces were strongly correlated in wild-caught shorebirds, indicating feces can be used to infer assimilated macronutrients. Breath $\delta^{13}C$ was $1.6 \pm 0.8\%e$ to $5.6 \pm 1.2\%e$ lower than bulk food sources, and breath C derived from lipids was estimated at $47.5\%$ (cereal) to $96.1\%$ (fish), likely underlining the importance of dietary lipids for metabolism. The findings validate the use of stable isotope values of feces and breath in isotopic assays to better understand the dietary needs of shorebirds.

Keywords Discrimination factors · Droppings · Blood · Feathers · Tissues

Introduction
Knowledge of diet is key to understanding species interactions, food web structure, nutrient physiology, and biogeochemical cycling (Martínez del Rio et al. 2009; Estes et al. 2011; Atwood et al. 2015; Hoenig et al. 2021). Dietary needs have been investigated using various conventional approaches, including the identification of dietary items ingested, their digestion and assimilation (Nielsen et al. 2018). The measurement of naturally occurring stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) of food and consumer tissues has become a widely used proxy for assessing diet (Fry 2006). Accordingly, proteinaceous tissues, including muscle, liver, and blood fraction are commonly collected from animals for $\delta^{13}C$ and $\delta^{15}N$ analyses (e.g., Hobson and Clark 1992a, b). However, catching wild animals is often difficult and harvesting tissue from captured individuals can be intrusive.

The analysis of excreta presents a non-intrusive means of inferring diet because such material often contains a large fraction of undigested material (Des Marais et al. 1980;
Podlesak et al. 2005; Salvarina et al. 2013). Generally, sampling excreta is straightforward, although individual- and population-based identification of each excreta is complicated by the problem of associating excreta with known individuals. Excreta do, of course, represent short-term dietary integration. Similarly, isotopic analysis of breath samples is relatively non-invasive compared to other techniques and represents recently metabolized diet, providing an indication of short-term energy pathways or stored macronutrients such as lipids or glycogen (Hwang et al. 2007; Hobson et al. 2009; Salvarina et al. 2013; McCue and Welch 2015). Feces and breath may serve for isotopic analyses in cases where either diet remains constant through time or a time series of material is available. However, to date, feces and breath have not been commonly used for dietary analyses of wild animals, and various aspects of their application require further examination, including the role of metabolic routing (Hobson et al. 2022) and potential isotopic discrimination between ingested diet and the resulting feces produced.

The full implementation of stable isotopic techniques to decipher diets requires knowledge of isotopic values of both dietary inputs as well as the isotopic discrimination, or isotopic difference, between diet and the tissues produced on that diet ($\Delta^{13}C$ and $\Delta^{15}N$). Also, knowledge of metabolic routing, or the preferential allocation of elements from macronutrients to ultimate tissue synthesis, is crucial but seldom considered (Podlesak et al. 2005; Hatch et al. 2002a; McCue and Welch 2015). The use of stable isotope measurements of tissues to infer diet is well established in birds (Hobson 2011; Karnovsky et al. 2012; Hoenig et al. 2021). However, determining trophic discrimination factors between animal diet and tissues is influenced by several considerations, including individual functional traits, resource availability, and isotope routing (Martínez del Río et al. 2009), and remains challenging especially when using bulk tissue analyses (Post 2002). While fecal samples have been used for research into the diets of wild birds (Podlesak et al. 2005; Salvarina et al. 2013), they have not been subjected to validation using isotopically controlled diets, and the assumption of previous researchers has been that $\Delta^{13}C$ and $\Delta^{15}N$ between the bulk food sources and the feces, at least for shorebirds, are negligible (Kuwae et al. 2008, 2012). Investigating the relative proportions of ingested but unassimilated dietary items and their macronutrient composition under controlled feeding conditions can determine the extent to which such an assumption is valid (Sponheimer et al. 2003) and how data from wild birds should be interpreted.

A necessary refinement to isotopic methodology for deciphering diets is the consideration of metabolic routing. Here, understanding the various metabolic pathways involved between diet and tissue synthesis is required. Tissue $\delta^{15}N$ analyses only provide information on dietary protein because lipids and carbohydrates contain little nitrogen. In contrast, carbon in tissues can enter by various pathways and be routed from all macronutrients into all tissues (Karasov and Martínez del Río 2007; Whiteman et al. 2012). Thus, ideally, trophic discrimination factors linking dietary macronutrients with consumer tissues should be known (Podlesak et al. 2005; Ben David et al. 2012; Hobson et al., 2022).

In this study, we investigated isotopic linkages between diet and feces, breath, feathers, and blood under diets differing in isotopic values of macronutrients using captive and wild shorebirds. First, captive Red-necked Stints, *Calidris ruficollis*, housed in a controlled tidal flat experimental mesocosm (Kuwae and Hosokawa 2000; Kuwae 2005) were used for a controlled-diet experiment. These birds were fed either a cereal- or fish-based diet of known bulk isotopic composition, and the isotope values of feces and breath measured. Second, we compared the isotope values of feces, blood, and feathers taken from captive birds held under standardized conditions in the mesocosm for 4 years. Third, associations between blood isotope values and those of feces were measured in a variety of wild shorebird species captured during migration on the east coast of Japan. The overall objective was to assess to what degree isotopic measurements of feces and breath samples could replace isotopic analyses based on blood or other tissues/samples to infer diet in shorebirds. The research allowed the isotopic consequences of metabolic routing of macronutrients, in particular, lipids, from diet to these tissues/samples to be assessed. Improved understanding of the natural variation of isotopic discrimination factors between individual traits such as body mass, irrespective of species, could allow a more general application of our findings to a broad spectrum of avian diet studies.

**Methods**

All work was conducted in accordance with applicable regulations in Japan (e.g., Protection and Control of Wild Birds and Mammals and Hunting Management Law, the Act on Welfare and Management of Animals) and all possible efforts were made to minimize stress on the shorebirds.

**Captive shorebirds in a tidal flat mesocosm**

**Mesocosm**

First-year juvenile Red-necked Stints were captured using mist nets at the Torinoumi intertidal flat (38°1.8′ N, 140° 54.9′ E), Miyagi, Japan, during their southward migration in September, 2013 ( Permit No. 1308061, Japanese Ministry of Environment, August, 6, 2013). Birds were transferred and reared (Registration No. 14B “a” 1910–1922, Kanagawa Prefecture, Japan, September 2, 2013) at the tidal
flat mesocosm, Port and Airport Research Institute (Kuwae and Hosokawa 2000; Kuwae 2005) (Fig. 1, see also Supplementary videos). The experiment was started with five acclimatized individuals (Individual bird ID: Birds A–E, see Supplementary Table 1). During the four-year experiment, captive birds fed on naturally occurring biofilm and macroinvertebrates in the mesocosm (Kuwae and Hosokawa 2000; unpublished data), plus a commercial cereal-based pellet diet.

In January 2019, a controlled-diet experiment was performed to examine how breath δ13C values would vary with changes in macronutrient content of the diet. Providing diets that differed in macronutrient composition (Table 1) enabled us to determine whether diet types affected trophic discrimination factors and metabolic routing (see Supplementary Materials for the macronutrient analyses of diets). Three of the focal birds (A–C) that had been held on the commercial cereal-based diet were isolated in an enclosure (inner cage, Fig. 1) and began receiving a fish-based pellet for a period of one week, during which breath samples were taken from birds on days one, two, and five of the experiment (see Supplementary Data File [Table 1_IFEF_breath]). After the switch period, birds were returned to the cereal-based pellet, and breath samples were taken from birds for four consecutive days. Food and water were available ad libitum throughout the acclimation and experimental periods, and no decrease in body masses occurred during the experiment.

Fecal sampling and preparation

Approximately 20 droppings excreted by 4–5 individuals (October 2013–May 2016: Birds A–E; September 2016–January 2017: Birds A–D) were collected each sampling day (n = 394 droppings), without an individual bird ID being assigned to each dropping (see Supplementary Data File [Table 2_IFEF_feces]). For the controlled-diet experiments, three individuals (Birds A–C) were isolated in an enclosure (inner cage, Fig. 1) and droppings (n = 77) collected after they received only a cereal-based pellet for two days, without an individual bird ID for each dropping (see Supplementary Data File [Table 1_IFEF_feces]) or only a commercial fish-based pellet (n = 20), without an individual bird ID for each dropping for another two days (see Supplementary Data File [Table 1_IFEF_feces]). For both cases, sampling commenced two days after isolation, allowing the droppings to reflect the particular pellet diet (Kuwae et al. 2008). Only fresh droppings were collected and immediately stored at −20 °C until drying.

| Table 1 | Macronutrient composition of the pellets used in the diet experiments with captive Red-necked Stints (Calidris ruficollis) |
|---------|-------------------------------------------------|
| Carbohydrates | Proteins | Lipids |
| % to total C | % to total C | % to total C |
| mean | SD | n | mean | SD | n | mean | SD | n |
| Cereal-based pellet | 45.7 | 1.5 | 5 | 31.0 | 0.5 | 5 | 23.4 | 1.8 | 5 |
| Fish-based pellet | 27.7 | 1.9 | 5 | 58.2 | 0.8 | 5 | 14.1 | 2.3 | 5 |
Droppings were pre-treated prior to stable isotope analyses to extract the fecal portion and remove isotopically fractionated metabolites, such as urea and ammonium, as well as carbonates (Kuwae et al. 2008, 2012). Feces (i.e., the fecal portion extracted from a dropping) and the two types of pellets used in the experiments described above were dried at 60 °C for 24 h, then ground to powder with a mortar and pestle. Subsamples of each powdered sample (ca. 5 mg) were placed in micro-tubes, mixed with a 1.4 ml 2:1 chloroform/methanol solution, centrifuged for 15 min at 1300×g, and the supernatant eliminated. The treatment was repeated four times. In the process, lipids and soluble carbohydrates (e.g., sugars) were also removed from the samples. The effect of the treatment on the isotope values of the two pellet types was also examined. Next, dropping samples were acidified using 1 M HCl to eliminate carbonates because of the sediment content in feces.

Feather sampling and preparation

Feathers were collected for deriving discrimination factors between different tissues/samples taken from individual birds experiencing similar conditions. As the collected feathers had dropped naturally, rather than being plucked, we were not able to match individual feather to the originating bird. Primary feathers (n = 4 in November, 2014; n = 4 in February, 2015) and breast feathers (n = 3 in November, 2014; n = 3 in February, 2015) were collected from the enclosure housing five second-year individuals (Birds A–E) during the period when all their juvenile feathers were being replaced by adult feathers (see Supplementary Data File [Table 3_IFEF_feather]). As such, we expected feather stable isotope values to reflect fully the provisioned diet treatments during the 4-years experiment. Feathers were stored at −20 °C until being treated by soaking in a 2:1 chloroform/methanol solution overnight, decanted and air-dried to remove surface oils. Then the feather samples were powdered with a mortar and pestle prior to analyses.

Blood sampling and preparation

Whole blood was taken from five individuals (Birds A–E) in June 2015. Approximately, 150 µL of blood was collected from the brachial vein in the wing with a sterilized 27G S.B. 3/4″ needle (Terumo, Tokyo, Japan) and transferred to a 250 µL heparinized capillary tube (Drummond Scientific Company, Broomall, Pennsylvania, USA, SKY-I KN3131665). The blood was centrifuged for 5 min at 7000 rpm (AcNo Light; Sagami Co., Ltd, Yokohama, Japan) to separate fractions of plasma and red blood cells, before being immediately transported to the laboratory and stored at −20 °C until drying (see Supplementary Data File [Table 3_IFEF_blood]). Plasma and red blood cell samples were dried at 60 °C for 24 h, then ground to powder with a mortar and pestle prior to analyses.

Breath sampling and preparation

Breath sampling was conducted by placing a bird in an air-tight plastic container that could be flushed and filled with CO₂-free air (using a hand pump to force air through a drierite and ascarite filter) before being isolated via stopcocks for three minutes, allowing bird breath CO₂ to accumulate (Fig. 2). Next, air from the container was subsampled through a sampling port using a gas-tight syringe and immediately introduced into an isotope-ratio mass spectrometer, described below. We confirmed that there was no contamination of breath samples with ambient CO₂ by a controlled experiment, in which measurements were taken with no birds in the containers and no CO₂ was detected.

Wild bird sampling

Twenty-six species of shorebirds, a total of 259 individuals (Supplementary Table 2), were captured by mist nets on the Torinoumi intertidal flat on their stopover during both northward and southward migrations (August 2012–November 2014) (Permit No. 120124001-42, 1302275, 1401301, 1503262, 1604181, and 1705171, Tohoku Regional Environment Office, Japanese Ministry of Environment). Following capture, each bird was tagged with an individually numbered leg band and aged (juvenile: until the first northward migration, adult: after the second southward migration) based on plumage characteristics and morphometric measurements (Higgins and Davies 1996). We confirmed that there were no recaptures based on the presence or absence of a leg band. Each captured bird was placed in a plastic case lined with

![Fig. 2 The device for breath sampling. a Airtight plastic container, b drierite and ascarite filter to generate CO₂-free air, c hand pump to force air through the filter, and d gas sampling port](image-url)
a plastic bag and left for <30 min to allow the collection of individual-based droppings. The fresh droppings were expected to reflect recent diet (minutes to hours, Kuwae 2007; Kuwae et al. 2008). Next, approximately 150 µL of blood was collected from a subsample of the captured birds, and part of the samples were centrifuged to separate fractions of plasma and red blood cells, using the same protocols described above, before the birds were released (see Supplementary Data File [Table 4_Torinoumi]). All the samples were transported on ice for <2 h and stored at −20 °C until drying, dried at 60 °C for 24 h, then ground to powder with a mortar and pestle prior to analyses.

**Stable isotope analyses**

Samples were weighed into tin foil capsules (ca. 1 mg), crushed and introduced into a zero blank autosampler under He flow. Next, samples were combusted at 1000 °C, and the gases separated in a Thermo Flash elemental analyzer (Thermo Instruments, Bremen, Germany) via a Conflo III device interfaced with a Thermo DELTA Plus Advantage continuous-flow isotope-ratio mass spectrometer at the Port and Airport Research Institute (Nagase, Yokosuka, Japan) to determine δ13C, δ15N, and the carbon/nitrogen elemental ratio (C/N ratio). Vienna Pee Dee Belemnite (VPDB) and atmospheric AIR were used as reference materials for δ13C and δ15N, respectively. Within-run laboratory calibration standards were L-Histidine [δ13C -VPDB = −10.18‰, δ15N-Air = −7.74‰; Shoko Co., Ltd., Minato-ku, Japan]; L-Alanine [δ13C -VPDB = −19.6‰, δ15N-Air = 10.1‰; Shoko Science Co., Ltd., Yokohama, Japan]; and Glycine [δ13C -VPDB = −32.3‰, δ15N-Air = 1.12‰; Shoko Science Co., Ltd.]). The long-term within-run analytical precision of the system was within ±0.2‰ for both δ13C and δ15N.

**Statistical analyses**

*Diet-controlled experiments in mesocosm.* We used a General Linear Model (GLM) to examine whether the δ13C of breath values differed depending on input diets, and whether δ13C values differed between the diets themselves. The model had the δ13C value as the response variable, with explanatory variables being the source of the measure, namely, bird breath (cereal diet), cereal (bulk), cereal (lipid removed), bird breath (fish diet), fish (bulk), and fish (lipid removed). We used a GLM to test for average differences in δ13C values between breath samples under different diets and between diets themselves.

Breath samples collected from the same birds may be considered statistically non-independent replicates, so, to examine the potential for any effects of pseudoreplication, we additionally used a General Linear Mixed-effects Model (GLMM; Bates et al. 2015) to analyze the δ13C data based on the breath samples alone (without including the samples from the diet items). The GLMM included random effects for individual ID and sample date, effectively controlled for different breath samples being taken from the same birds, analogous to a repeated measures design. The residual SD from the GLMM had a value of 0.93, higher than the bird ID random effect of 0.33, and the date SD value with a 0.82, meaning samples taken from the same bird were more variable than samples taken from different birds or from a different date. This pattern is consistent with the high C turnover in breath (Podlesak et al. 2005). Thus, we retained the GLM approach, with the understanding that some interdependence may exist between some replicates, but it was not sufficient to alter the main conclusions (that birds on different diets have different breath δ13C values).

**Wild-caught birds.** The δ13C and δ15N values of feces and plasma from the wild-caught birds and GLMMs were used to examine whether these δ13C and δ15N values varied by age of bird (adult vs juvenile, categorical variable) and ln-transformed body mass. These GLMMs included species as a random effect to account for non-independence in the data that may have occurred, as individuals within the same species can be expected to have similar isotope values, given they would likely be feeding sympatrically and simultaneously. We did not elaborate on the relationship between feces and blood cells/whole blood because the timing of food reflected was different, that is, feces reflect the diet from minutes to hours before sample collection, and plasma reflects the diet integrated from days before; however, whole blood and blood cells reflect the diet integrated from weeks before (Supplementary Table 4). Thus, comparisons between feces and plasma are reasonable given their comparable turnover rates. In addition, we used GLMMs with species as a random effect to examine whether isotopic discrimination factors (i.e., Δ13C and Δ15N) between diet and blood and feces varied by age, body mass, and δ13C and δ15N values in feces.

Heteroscedasticity and normality of errors of all GLMMs were assessed according to Crawley (2005). A Gaussian error distribution proved the best model structure and was used for the analyses. Selection of candidate models was based on the principle of parsimony (Burnham and Anderson 2002). We first fitted the global model with all explanatory variables, then used Akaike’s Information Criterion (AIC) to select the most parsimonious model (the smallest AIC) by both backward and forward stepwise variable selection. All statistical analyses were performed using R 4.1.1 (R Core Team 2021).
Results

Diet-controlled experiments in mesocosm

Food isotope values

As expected, δ¹³C values of food items that had undergone lipid removal treatment were significantly higher than those without treatment (Wilcoxon rank sum test, $P \leq 0.001$, see Table 2, Fig. 3). The C/N ratio with lipids removed was significantly lower than those without lipid removal (Wilcoxon rank sum test, $P \leq 0.001$). Further, as expected, there was no significant difference between the δ¹⁵N of food with and without lipid removal ($P=0.739$).

Feces isotope values

Average body masses of three individuals (Birds A–C) fed cereal-based pellets in September, 2018, ranged from 23.7 to 24.9 g, compared to 27.1 to 28.5 g for the same three birds with fish-based pellets in April, 2017, with 0.6 ± 0.4 g (mean ± SD) as the individual body mass ranges during the experiment.

The δ¹³C values of feces closely resembled those of diets with lipids removed (Table 2); the δ¹³C between feces and food with lipid removal was −0.2 ± 0.5‰ (cereal) and 0.1 ± 0.3‰ (fish), with no significant difference between them ($P=0.432$ and $P=0.083$, respectively). Also, the δ¹⁵N values of feces closely resembled those of diets, showing the Δ¹⁵N between feces and food with lipid removal being −0.6 ± 0.6‰ (cereal) and −0.5 ± 0.5‰ (fish); however, there was a significant difference between them ($P=0.001$ and $P=0.015$, respectively).

Breath isotope values

The average body mass of the three individuals (Birds A–C) during the breath experiment ranged from 24.3 to 25.3 g, with 0.5 ± 0.3 g (mean ± SD) the individual body mass range during the experiment, and 0.3 ± 0.2 g (mean ± SD) the difference in mass between the start and the end of the experiments.

Table 2: Stable isotope values (δ¹³C and δ¹⁵N) of fecal and breath samples collected from five captive Red-necked Stints (Calidris ruficollis) fed either a cereal-based pellet or a fish-based pellet during diet-controlled experiments using a mesocosm

|                         | δ¹³C        | δ¹⁵N        | C/N ratio |
|-------------------------|-------------|-------------|-----------|
|                         | mean SD n   | mean SD n   | mean SD n |
| **Cereal-based pellets**|             |             |           |
| Bulk food (a)           | −26.7 0.2 10| 6.2 0.1 10  | 11.4 0.3 10|
| Food with lipid removal (b) | −25.1 0.1 10| 6.2 0.2 10  | 9.2 0.2 10 |
| Dietary lipids (c)      | −31.8       |             |           |
| Depletion of lipids to other macronutrients (c−b) | −6.7 | | |
| Feces (d)               | −25.3 0.5 77| 5.6 0.6 77  | 6.9 2.6 77 |
| Feces–food discrimination factor (Δ, d–b) | −0.2 0.5 | −0.6 0.6 |
| Breath (e)              | −28.3 0.8 25|             |           |
| Breath–bulk food discrimination factor (Δ, e–a) | −1.6 0.8 | | |
| Breath–food lipids discrimination factor (Δ, e–c) | 3.5 | | |
| **Fish-based pellets**  |             |             |           |
| Bulk food (f)           | −20.7 0.1 5 | 10.7 0.2 5  | 6.2 0.2 5 |
| Food with lipid removal (g) | −19.7 0.1 5 | 10.5 0.1 5  | 5.2 0.3 5 |
| Food lipids (h)         | −26.5       |             |           |
| Depletion of lipids to other macronutrients (c−b) | −6.8 | | |
| Feces (i)               | −19.6 0.3 20| 10.0 0.5 20| 3.1 0.5 20|
| Feces–food discrimination factor (Δ, g–f) | 0.1 0.3 | −0.5 0.5 |
| Breath (j)              | −26.3 1.2 16|             |           |
| Breath–bulk food discrimination factor (Δ, j–i) | −5.6 1.2 | | |
| Breath–food lipids discrimination factor (Δ, j–h) | 0.3 | | |

Bulk food values represent the input diets, and discrimination factors (Δ) refer to the differences between diets and fecal or breath samples. Dietary lipid δ¹³C values were calculated from the bulk diet δ¹³C values with lipids and with lipids removed and also had the compositional analyses of the bulk diets (Table 1). Note that multiple breath or fecal samples were taken from each individual bird (see “Methods”). C/N ratio indicates the carbon/nitrogen elemental ratio.
The δ¹³C values in breath samples differed between birds receiving either a cereal-based or a fish diet (Table 2; Fig. 3). The GLM indicated that δ¹³C values differed widely among the six sources (Likelihood ratio test: $\chi^2 = 40.0$, df = 6, $P < 0.001$). Breath δ¹³C values for birds on both diets were lower than their respective dietary inputs (Fig. 3). The differences (Δ¹³C) between breath and bulk food were $-1.6 \pm 0.8\%e$ (cereal) and $-5.6 \pm 1.2\%e$ (fish), and between breath and food with lipid removal was $-3.2 \pm 0.8\%e$ (cereal) and $-6.6 \pm 0.5\%e$ (fish).

We were able to estimate the lipid δ¹³C values associated with diet because we measured the bulk diet δ¹³C values with lipids and with lipids removed and also had the compositional analyses of the bulk diets (cereal = 23.4% lipid, fish = 14.1% lipid). This resulted in mean estimates for cereal food lipid as $-31.8\%e$ and $-26.5\%e$ for the fish diet. Assuming that diet-breath isotopic discrimination during the oxidation of macronutrients in the tricarboxylic acid cycle (TCA) cycle is zero, and that birds were metabolizing only dietary lipids into breath CO₂, then the corresponding mean Δ¹³C for dietary lipid to breath for the cereal diet would be 3.5‰ and 0.3‰ for the mean Δ¹³C for dietary lipid to breath in the fish diet. In addition, if birds were metabolizing all the macronutrients, the lipid content of breath is estimated to be 47.5% for the cereal-based diet and 96.1% for the fish-based diet.

**Diet studies in mesocosm**

Average body mass of captive birds during the 4-year experiment ranged from 25.9 ± 2.1 g to 28.5 ± 3.2 g (mean ± SD, Supplementary Table 2), with a clear seasonal fluctuation, the highest being around June and the lowest around December (Kuwae et al., unpublished).

The δ¹³C, δ¹⁵N, and C/N ratios for feces, blood, and feathers are shown in Table 3. The Δ¹³C and the Δ¹⁵N between plasma and feces were $-0.7\%e$ and $2.7\%e$, respectively. The Δ¹³C and the Δ¹⁵N between blood cell and feces were 1.5‰ and 2.3‰, respectively. The Δ¹³C and the Δ¹⁵N between primary feathers and feces were 3.0‰ and 3.9‰, respectively, and breast feathers and feces were 2.2‰ and 3.7‰, respectively.

**Isotope values in wild-caught shorebirds**

The average fecal δ¹³C and δ¹⁵N values from the 26 species of shorebirds were $-20.2 \pm 2.8\%e$ and $8.7 \pm 2.3\%e$, respectively (Table 4, see Supplementary Fig. 1 for each species value). The δ¹³C and δ¹⁵N values of feces were both positively correlated with the body mass of individual birds (Fig. 4). In GLMMs for both isotopes, the residual SD was larger than the SD for the random effect of species (Table 5), indicating that the within-species variance was larger than across species (Supplementary Fig. 1).

Within-individual (paired) δ¹³C and δ¹⁵N of feces were significantly different from those of plasma and whole blood ($P < 0.05$) but not significantly different from those of blood cells ($P = 0.254$ and $P = 0.056$, respectively) (Table 4). The within-individual Δ¹³C and the Δ¹⁵N between plasma and feces were $0.6 \pm 1.6\%e$ and $2.7 \pm 1.1\%e$, respectively, whereas those between whole blood and feces were $1.5 \pm 3.3\%e$ and $1.2 \pm 1.7\%e$, respectively. Within-individual δ¹³C and δ¹⁵N of feces showed strong correlations with those of plasma (Fig. 5).

Isotopic discrimination factors were associated with diets of birds, and did not vary with body mass or age class. GLMM results supported that within-individual Δ¹³C and Δ¹⁵N between feces and plasma/blood cells were negatively correlated with δ¹³C and δ¹⁵N values of feces (Table 5 and Supplementary Figs. 2 and 3). In contrast, the Δ¹³C and the Δ¹⁵N between feces and whole blood were not correlated with δ¹³C and δ¹⁵N of feces. Stepwise variable selection in GLMM results did not indicate that any of the Δ¹³C and Δ¹⁵N values were associated with body mass or age class of sampled birds.

![Fig. 3 Stable isotope values (δ¹³C) of breath samples collected from three captive Red-necked Stints (Calidris ruficollis), along with cereal- and fish-based input diets during controlled experiments. Box plot extends from lower quartile to the upper quartile, with median values as a solid bar, and whiskers extend to 1.5× the interquartile range. Blue points and errors indicate predicted means from a General Linear Model (GLM; see Results). Letters above each box plot denote groups that are statistically different based on least-squared means. Colored data points indicate individual birds in the experiment.](image-url)
Discussion

Feces samples for diet studies

Our results showed that fecal samples represent a means for reconstructing short-term diets in shorebirds without a need to capture birds. Given both Δ13C and Δ15N between feces and lipid-extracted food are small (−0.6‰ to 0.1‰) in the captive diet-controlled experiment, feces can be used to identify the bulk food sources of ingested proteinaceous tissues and non-soluble carbohydrates for shorebirds (Table 2). Also, feces are advantageous because defeation rates of shorebirds are high, at least during the migratory stopover period when rapid refueling is required (Kuwae et al. 2008; Canham 2020), and thus feces reflect on-site food sources and reducing the carry-over effect from former staging sites (Dietz et al. 2010). Moreover, the high fecal production rate of shorebirds may be largely responsible for the strong correlations between isotope values of bulk diet and feces.

Table 3 Stable isotope values (δ13C and δ15N) of fecal and breath samples collected from captive Red-necked Stints (Calidris ruficollis) feeding on diets of intertidal biofilm and invertebrates, supplemented with cereal-based pellets during diet-non-controlled experiments using a mesocosm

|                          | δ13C (‰) |           | δ15N (‰) |           | C/N ratio |           |
|--------------------------|----------|-----------|-----------|-----------|-----------|-----------|
|                          | mean     | SD        | n         | mean      | SD        | n         | mean      | SD        | n         |
| Feces                    |          |           |           |           |           |           |           |           |           |
| All (a)                  | −24.3    | 1.0       | 394       | 7.0       | 0.7       | 394       | 5.6       | 1.8       | 394       |
| During plasma turnover period (b)* | −24.5    | 0.3       | 18        | 7.3       | 0.5       | 18        | 6.0       | 1.6       | 18        |
| During blood cell turnover period (c)** | −24.3    | 0.3       | 38        | 7.1       | 0.5       | 38        | 6.1       | 1.7       | 38        |
| Blood                    |          |           |           |           |           |           |           |           |           |
| Plasma (d)               | −25.2    | 0.3       | 5         | 10.1      | 0.2       | 5         | 6.2       | 0.1       | 5         |
| Plasma–feces discrimination factor (Δ, d–b) | −0.7     | 2.7       |           |           |           |           |           |           |           |
| Blood cell (e)           | −22.8    | 0.3       | 5         | 9.4       | 0.3       | 5         | 3.6       | 0.1       | 5         |
| Blood cell–feces discrimination factor (Δ, e–c) | 1.5      | 2.3       |           |           |           |           |           |           |           |
| Feather                  |          |           |           |           |           |           |           |           |           |
| Primary (f)              | −21.3    | 0.3       | 8         | 10.9      | 0.3       | 8         | 3.5       | 0.0       | 8         |
| Breast (g)               | −22.2    | 0.1       | 6         | 10.7      | 0.3       | 6         | 3.6       | 0.1       | 6         |
| Primary feather–feces discrimination factor (Δ, f–a) | 3.0      | 3.9       |           |           |           |           |           |           |           |
| Breast feather–feces discrimination factor (Δ, g–a) | 2.2      | 3.7       |           |           |           |           |           |           |           |

Discrimination factors (Δ) refer to the differences between feces and breath or blood samples. C/N ratio indicates the carbon/nitrogen elemental ratio. Feather samples may have come from the same birds

*Sampled on the same day as the plasma sampling considering the half-life of plasma (up to days)
**Sampled over two months prior to the blood cell sampling considering the half-life of blood cells (up to weeks)

Table 4 Stable isotope values (δ13C and δ15N) of fecal and blood samples collected from wild-caught shorebirds at Torinoumi tidal flat, Japan

|                          | δ13C (‰) |           | δ15N (‰) |           | C/N ratio |           |
|--------------------------|----------|-----------|-----------|-----------|-----------|-----------|
|                          | mean     | SD        | n         | P         | mean      | SD        | n         | P         |
| Feces (a)                |          |           |           |           |           |           |           |           |
| Blood                    |          |           |           |           |           |           |           |           |
| Plasma (b)               | −20.2    | 2.8       | 258       | 8.7       | 2.3       | 259       |           |           |
| Individual-based plasma–feces discrimination factor (Δ, b–a) | −19.5    | 3.0       | 47        | 11.7      | 2.1       | 48        |           |           |
| Blood cell (c)           | −19.8    | 3.2       | 54        | 2.7       | 1.1       | 47        | <0.001    |           |
| Individual-based blood cell–feces discrimination factor (Δ, c–a) | 0.6      | 1.6       | 44        | 0.021     | 9.6       | 2.1       | 59        |           |
| Whole blood (d)          | −17.6    | 4.3       | 29        | 0.5       | 1.8       | 58        | 0.056     |           |
| Individual-based whole blood–feces discrimination factor (Δ, d–a) | 1.5      | 3.3       | 28        | 1.2       | 1.7       | 27        | 0.001     |           |

Discrimination factors (Δ) refer to the differences between feces and blood samples

Discussion
When isotopically fractionated nitrogenous metabolites, lipids, and soluble carbohydrates (sugars) were removed from fecal samples by pre-treatment, the δ13C and δ15N of feces in our study primarily reflected dietary proteins and non-soluble carbohydrates, such as celluloses. The smaller Δ13C and Δ15N values between feces and food with lipid removal, relative to bulk food, clearly indicate that the δ13C and δ15N of the treated feces better represented the δ13C and δ15N values of ingested proteins and non-soluble carbohydrates.

Our finding that isotope values of feces were similar to lipid-extracted bulk food sources was also supported from the results of Δ13C and Δ15N comparisons between feces and feathers for the captive diet non-controlled experiment, and by the Δ13C and Δ15N comparisons between feces and blood in the wild-caught birds and captive birds during the mesocosm trials (Tables 3 and 4). All these trophic discrimination factors fell within previously reported ranges for the Δ13C and the Δ15N between the diet and tissues/samples with lipid removal (e.g., Peterson and Fry 1987; Hobson and Clark 1992a, b).

The δ13C and δ15N of feces have been commonly assumed to be less useful for dietary analyses as feces per se are an aggregation of materials that were either not digested or assimilated. However, in a shorebird study by Kuwae et al. (2008) of the energy budget paired with stable isotope analyses, ca. 25% of the organic matter in the diet was included in the excreta, with no isotope discrimination between this material and the ingested food. Hence, we infer that the macronutrient composition (proteins, carbohydrates, and lipids) of undigested organic matter excreted as feces is not affected by isotope discrimination during digestion and absorption in the digestive tracts of shorebirds (Fry 2006). Similarly, Salvarina et al. (2013) reported no significant difference in δ13C and δ15N values among feces with lipid removal treatment, feces without lipid removal treatment, and diet in captive bats (0.1 ± 0.8‰ for δ13C and 1.5 ± 1.5‰ for δ15N). In contrast, differences in Δ13C and Δ15N between excreta and food have been reported for songbirds (Podlesak et al. 2005) and rodents (Hwang et al. 2007), although no pre-treatment for excreta was applied in these latter studies. Hence, our pre-treatment that removed isotopically fractionated metabolites, such as urea and ammonium, lipids and soluble carbonates, would support its utility for dietary analyses as feces per se are an aggregation of materials that were either not digested or assimilated.

Previous studies on omnivores have suggested that the use of feces incurs the possible bias that less digestible food sources are overrepresented and more digestible food sources underrepresented (Sponheimer et al. 2003; Kuwae et al. 2008, 2012). The commercial pellets used for the present study were formulated for growing birds and contained more digestible food sources compared to wild diets, suggesting that effects of possible overrepresentation of less digestible parts should have been minimized. Overall, while feces cannot provide insight on long-term assimilated diet, we argue that for shorebirds with rapid ingestion and high fecal production rates, fecal isotopic analyses provide a close approximation to recently assimilated diet.

Establishing appropriate trophic discrimination factors between diet and consumer tissues/samples is critical for dietary reconstructions using stable isotope measurements. However, potentially confounding parameters, including
metabolic rate, nutritional quality, tissue type, metabolic routing and degree of fasting, also need to be considered (Hobson et al. 1993; Caut et al. 2008a, b, 2009). For our two experimental diets, we found negligible Δ13C and Δ15N values between diet and feces. These results are encouraging as they suggest that δ13C and δ15N values of suitably treated feces can be used as a proxy for those values in bulk diet. Regardless, before feces can be used to provide direct evidence of assimilation, consistency of stable isotope values should be confirmed between feces and consumer tissues formed through assimilated diet. The latter could be done by sampling feces and blood, as demonstrated here.

Our data from wild-caught birds showed a positive relationship between the δ13C and δ15N values of the feces and shorebird body mass, a pattern that was consistent across species (Fig. 4). Possibly, large-bodied birds are more able than smaller counterparts to feed on larger prey that are generally from higher trophic positions (with corresponding higher δ13C and δ15N values; Kuwae et al. 2012). Our result that the within-species isotopic variance, that is individual-level variance, was larger than across species (Supplementary Fig. 1) underscores the need to consider individual functional traits, such as body mass, as well as species-level traits.

The observed negative relationships between within-individual Δ13C and Δ15N between feces and plasma/blood cells with δ13C and δ15N values of feces needs further study (Table 5 and Supplementary Figs. 2 and 3). However, if we

### Table 5

| Response variable | Variable | Estimate | SE   | P       | n  |
|-------------------|----------|----------|------|---------|----|
| (R² value of the best model)  
δ13C of feces (0.48) | Intercept | −26.02 (+) | 2.79 | <0.0001 | 238 |
|                    | ln(Body mass) | 1.13 | 0.65 | 0.090 |    |
|                    | Species RE (SD) | 2.20 | | | |
|                    | Residual (SD) | 2.37 | | | |
| δ15N of feces (0.37) | Intercept | 1.15 (+) | 2.30 | 0.620 | 239 |
|                    | ln(Body mass) | 1.74 | 0.55 | 0.003 |    |
|                    | Species RE (SD) | 1.39 | | | |
|                    | Residual (SD) | 2.19 | | | |
| Δ13C between plasma and feces (0.29) | Intercept | −3.45 (+) | 1.59 | 0.035 | 44 |
|                    | δ13C of feces | −0.19 (+) | 0.08 | 0.018 |    |
|                    | Species RE (SD) | 0.67 | | | |
|                    | Residual (SD) | 1.39 | | | |
| Δ13C between blood cell and feces (0.66) | Intercept | −13.62 (+) | 2.71 | <0.001 | 52 |
|                    | δ13C of feces | −0.64 (+) | 0.13 | <0.001 |    |
|                    | Species RE (SD) | 2.25 | | | |
|                    | Residual (SD) | 2.04 | | | |
| Δ13C between whole blood and feces (0.19) | Intercept | 1.54 (+) | 0.88 | 0.152 | 28 |
|                    | Species RE (SD) | 1.47 | | | |
|                    | Residual (SD) | 3.06 | | | |
| Δ15N between plasma and feces (0.63) | Intercept | 4.89 (+) | 0.58 | <0.001 | 47 |
|                    | δ15N of feces | −0.27 (+) | 0.06 | <0.001 | 47 |
|                    | Species RE (SD) | 0.83 | | | |
|                    | Residual (SD) | 0.79 | | | |
| Δ15N between blood cell and feces (0.55) | Intercept | 4.27 (+) | 0.93 | <0.001 | 58 |
|                    | δ15N of feces | −0.45 (+) | 0.93 | <0.001 | 58 |
|                    | Species RE (SD) | 1.18 | | | |
|                    | Residual (SD) | 1.41 | | | |
| Δ15N between whole blood and feces (0.05) | Intercept | 1.21 (+) | 0.37 | 0.037 | 27 |
|                    | Species RE (SD) | 0.37 | | | |
|                    | Residual (SD) | 1.65 | | | |

Discrimination factors (Δ) refer to the differences between feces and blood samples. Explanatory variables for each model were selected via a bothways stepwise procedure from an initial global model that included ln(Body mass), age (juvenile/adult), and Species as a Random effect (RE). Initial global models for discrimination factors (Δ) also included stable isotope values of feces. n refers to the number of feces samples used in each model.
assume that $\delta^{13}C$ and $\delta^{15}N$ values of feces represent those of diet, the same relationship has been found for other consumers (Hobson and Clark 1992b; Caut et al. 2008a, b, 2009, 2010). One hypothesis that might explain this negative relationship relates to $\delta^{15}N$ decreasing as dietary protein quality (i.e., the degree to which the diet meets the amino acid requirements of the consumer) increases, and, therefore, $\Delta^{15}N$ decreases with the trophic position ($\delta^{15}N$) of consumers (Robbins et al. 2005; Caut et al. 2010; but see Pearson et al. 2003).

A clear positive relationship was found between the within-individual $\delta^{13}C$ and $\delta^{15}N$ of feces and plasma for wild-caught shorebirds, independent of age, body mass, and species (Fig. 5). To examine the robustness of this correlation depending on defecation rate, digestibility efficiency, and degree of omnivory, as suggested, we still have to establish the correlation between within-individual $\delta^{13}C$ and $\delta^{15}N$ of feces and plasma across a variety of habitats. However, overall, given that feces sampling is easy, cost effective and non-intrusive, the $\delta^{13}C$ and $\delta^{15}N$ of feces appear practical for short-term diet inferences.

### Breath samples for diet studies

We found that $\Delta^{13}C$ between breath CO$_2$ and bulk diets were significantly negative for both the cereal-based (carbohydrate-rich) pellet ($-3.2$ to $-1.6\%e$) and fish-based (protein-rich) pellet ($-6.6$ to $-5.6\%e$); that is, the $\delta^{13}C$ of breath CO$_2$ was lower than the bulk food sources (Fig. 3). However, while this result seems contrary to previous studies (McCue and Welch 2015; Whiteman et al. 2012), evaluating which substrate was likely being used for metabolism is critical (Hobson et al. 2022). Fasting animals that switch to metabolize endogenous lipids usually show declines in the $\delta^{13}C$ of breath CO$_2$ because lipids are typically depleted in $^{13}C$ by 2–8%e compared to other macronutrients (DeNiro and Epstein 1978; Peterson and Fry 1987; Hatch et al. 2002b; McCue and Welch 2015). Indeed, the $\delta^{13}C$ of lipids of the food sources, estimated from the results of the macronutrient composition of the pellets (Table 1) and the $\delta^{13}C$ of the bulk food sources and with lipid removal (Table 2), was $-32.2\%e$ for the cereal-based pellet and $-26.8\%e$ for the fish-based pellet, with $6.7\%e$ and $6.8\%e$ depletion, respectively, compared to the other macronutrients. However, in our case, endogenous lipids and proteins stored in the body were unlikely to have been oxidized and reflected in the $\delta^{13}C$ of breath CO$_2$ given that food and water were available ad libitum and body mass did not decrease during the experimental periods. Thus, dietary lipids were likely to have been preferentially oxidized in our study. The lipid content of breath was estimated to be 47.5% and 96.1% for the cereal- and fish-based diets, respectively. The subsequent estimate of mean $\Delta^{13}C$ between dietary lipids from the cereal diet and breath CO$_2$ of $+3.5\%e$ was much closer to the results of previous studies that estimated little discrimination between metabolized substrate and breath CO$_2$ (Voigt et al. 2008). The higher mean $\Delta^{13}C$ between dietary lipids from the cereal diet and breath CO$_2$ of $+3.5\%e$ reflected use of other macronutrients in addition to lipids for that dietary group. Why lipids were used more than other macronutrients for energy metabolism may be explained by the experiment not being performed during either the pre-migration or molting period when protein demand is high. Nevertheless, our results underline the need to understand which macromolecular substrate is being metabolized when trying to interpret breath CO$_2$ $\delta^{13}C$ values (Hobson et al. 2022).

Our findings suggesting selective metabolism of lipids relative to other macronutrients underscore the importance of considering macronutrient routing vs. food sources per
se as well as individual ecophysiology (Hobson et al. 2022). In particular, migratory birds vary considerably in their use of dietary carbohydrates, lipids, and proteins from local food versus endogenous stores at stopover and refueling sites (Hobson et al. 2009; Podelsak et al. 2005; Podelsak and McWilliams 2006). In future research, the additional use of the respiratory quotient (RQ) as a means of confirming which macronutrients are being metabolized to CO2 will fundamentally advance these captive studies, as a RQ of 1 indicates carbohydrate metabolism, whereas a RQ of 0.7 indicates pure lipid metabolism when no proteins are involved (Brody 1999). Also, there is need to evaluate isotopic discrimination factors between macronutrients and breath CO2 and breath δ13C during a fasted and fed state using 13C labeling experiments to establish appropriate trophic discrimination factors for dietary reconstruction.

In summary, stable isotopes of carbon (δ13C) and nitrogen (δ15N) derived from feces and breath samples offer potential as non-destructive tools to assess diets and better understand the nutritional needs of staging shorebirds and, possibly, other animals. The δ13C and δ15N values from feces with lipid removal provide a valid proxy for ingested proteinaceous tissues and non-soluble carbohydrates. Similarly, stable isotope values in plasma and feces were strongly correlated, indicating feces in addition to plasma may also be used to infer assimilated macronutrients. These results suggest that the isotopic reconstruction of diets of shorebirds can be greatly simplified and made less invasive.

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