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Simple ways to calculate stable isotope discrimination factors and convert between tissue types.

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Abstract

Traditional methods to determine stable isotope discrimination factors (Δ) between an animal's diet and tissue(s) are costly and time-consuming. Consequently, data are only available for relatively few species and are completely absent from some orders, including parrots (Order: psittaciformes). We present simple and cost-effective methodologies for establishing discrimination factors and converting between tissue types. We investigated $\Delta^{13}C_{diet\text{-}feather}$ and $\Delta^{15}N_{diet\text{-}feather}$ values for the kea parrot *Nestor notabilis* by comparing the isotope values from feathers of a population held under their regular conditions at a local zoo, with the $\delta^{13}C$ and $\delta^{15}N$ values from their weekly diet of >30 food items. We mathematically controlled for dietary elemental concentration, and the potential impacts of metabolic routing, the exclusive consumption of preferred foods, and the large-scale consumption of self-sourced plants and invertebrates; resulting in $\Delta^{13}C_{diet\text{-}feather}$ = 4.00‰ ± 0.03 and $\Delta^{15}N_{diet\text{-}feather}$ = 3.10‰ ± 0.20. We also determined regression equations for predicting feather $\delta^{13}C$ and $\delta^{15}N$ values from whole blood values by sampling simultaneously grown feathers and blood from wild kea nestlings. These are the first feather-blood discrimination equations determined for terrestrial birds in the wild. Our $\delta^{13}C$ feather-blood discrimination equation was similar to an equation developed for use across marine birds; however, the $\delta^{15}N$ feather-blood discrimination equation for marine birds consistently underestimated kea feather $\delta^{15}N$ values. These methodologies, while developed for use in birds, can easily be applied to other animal classes given the appropriate selection of tissues.

Introduction

Natural abundance stable isotope ratios, in particular those of carbon (^{13}C / ^{12}C, represented by $\delta^{13}C$) and nitrogen (^{15}N / ^{14}N, or $\delta^{15}N$), have a broad range of ecological applications. The $\delta^{13}C$ and $\delta^{15}N$ values obtained from an animal's tissue reflect those of its diet, and can be used to investigate dietary shifts (Phillips & Eldridge 2006), the diet of difficult-to-track species (Borrell et al. 2013), niche width (Layman et al. 2007), food web structure (Hussey et al. 2014), and migration routes

(Hobson 1999).

Sampling tissues with differing metabolic turnover rates enables researchers to examine diet over days (plasma, liver), weeks/months (blood, muscle) or years (bone collagen; Dalerum & Angerbjörn 2005). Tissues which can be sampled non-destructively (e.g., blood, fur and muscle), can be sampled repeatedly, and are suitable when investigating rare species. For avian ecologists, blood and feathers are increasingly becoming the "tissues of choice" (Bearhop et al. 2002). Blood reflects a bird's diet over the previous two to six weeks depending on the species (Hobson & Clarke 1993; Hobson & Bairlein 2003), whereas feather is made of keratin which remains metabolically inert after synthesis, and reflects diet at the time of feather growth, perhaps months earlier (Hobson & Clarke 1992). This temporal disjunct makes simultaneous sampling of blood and feathers particularly useful, as it allows investigations of diet over two separate time periods. For example, dual-tissue sampling from a nesting female allows her breeding (blood) and moulting (feather) diets to be compared.

When food is incorporated into animal tissue, light and heavy isotopes react differently due to differences in mass and bond strength. Nitrogen atom bonds are formed and broken during amino acid synthesis which leaves the product amino acids enriched in ^{15}N (Chikaraishi et al. 2009). Thus, fewer ^{15}N atoms are excreted resulting in ^{15}N enrichment in consumer tissues across progressively higher trophic levels of a food-chain (DeNiro & Epstein 1981). The difference between the dietary isotope value and the resulting tissue value is referred to as the discrimination factor (denoted by Δ). Discrimination factors vary both between species and within, depending on factors such as tissue type (Cherel et al. 2014); dietary protein quality (Robbins et al. 2010); and the consumer's growth rate (Martínez del Rio & Wolf 2005). Dietary protein may also be preferentially routed into the synthesis of proteinaceous tissues, such as feathers and blood (Voigt et al. 2008), disproportionately affecting $\Delta^{13}C$ and $\Delta^{15}N$ values. Some metabolic routing of dietary protein into tissue synthesis certainly occurs because essential amino acids can only be obtained from dietary protein. Moreover, because it is metabolically more efficient to incorporate non-essential dietary amino acids directly from the diet than to create new ones, additional routing is highly likely. Yet few

studies attempting to establish accurate discrimination factors take metabolic routing into account (but see Podlesak & MacWilliams 2006; Kurle et al. 2014).

Discrimination factors are typically determined by 'constant diet experiments' (e.g., Bearhop et al. 2002; Kurle et al. 2013) where an animal is fed a controlled diet of very few (usually no more than two or three) food items for a period of months. However, these experiments are costly, time-consuming and may not be ethical, depending on the species concerned and their natural feeding behaviour. Consequently, discrimination factors have been established for relatively few species. Values from a related species or generic values from reviews (e.g., Δ^{15}N = 3.4‰ per trophic level, Δ^{13}C = 0‰ to 1‰ per trophic level; Post 2002) are commonly substituted. However, even within birds, Δ^{13}C and Δ^{15}N values range widely (-1.5‰ to 4.3‰ per trophic level; and 0.2‰ to 5.6‰ per trophic level, respectively; Hobson & Clarke 1992; Pearson et al. 2003; Kempster et al. 2007; Federer et al. 2012), making this practice questionable (Caut et al. 2009). Certainly, because Δ is tissue dependent (Wolf et al. 2009), it is inappropriate to apply generic values to all tissues equally. The accuracy of discrimination factors is of central importance to isotope ecologists as they are used to calculate trophic position and relationships (Post 2002); and also form an integral part of the equations used in stable isotope mixing models where a discrepancy of just 1‰ can greatly alter the estimated contributions of dietary sources (Bond & Diamond 2011). Parrots (Order: Psittaciformes) are among the orders for which discrimination factors have not yet been established. Here, we devised a methodology to determine stable carbon and nitrogen isotope discrimination factors for kea *Nestor notabilis*, an endangered parrot for which constant diet experiments are inappropriate due to their extremely varied natural diet and high captive enrichment requirements (Gyula K. Gajdon, pers. comm.).

An animal's various tissues differ in their diet-to-tissue discrimination factors (DeNiro & Epstein 1981). Therefore, δ^{13}C and δ^{15}N values from blood and feathers can only be directly compared if these differences in discrimination factors are accounted for (Dalerum & Angerbjörn 2005). Traditionally, tissue differences have been established in constant diet experiments at the

same time as diet-to-tissue discrimination factors. However, it is also possible to establish tissue-specific differences in discrimination by sampling tissues synthesised at the same time. For example, blood and currently growing feathers, which can easily be sampled from either moulting birds, or nestlings attaining their fledgling plumage, will have been produced over a similar time scale (Quillfeldt et al. 2008). Therefore the difference in discrimination between feathers and blood can be investigated and species-specific regression equations calculated to allow direct comparisons between tissues. To date, these methods have only been applied by marine bird ecologists, who have recorded differences in $\Delta^{13}C$ and $\Delta^{15}N$ values between blood and feather from >30 species (see Cherel et al. 2014); whereas, for terrestrial birds, these differences have been entirely established during constant diet experiments and are available for a mere six species (Hobson & Clarke 1992; Hobson & Bairlein 2003; Pearson et al. 2003; Kempster et al. 2007; Kurle et al. 2013). Here we present the first data on the differences between $\Delta_{diet\text{-}feather}$ and $\Delta_{diet\text{-}blood}$ values for a parrot, and the first use of this methodology for terrestrial birds. We also investigate the utility of regression equations derived for use in predominantly carnivorous, marine bird taxa (Cherel et al. 2014) to convert between the stable isotope values from blood and feathers from an omnivorous parrot.

Methods

Determination of discrimination factors for feathers

We obtained 18 kea feathers for carbon and nitrogen stable isotope analysis from a population held at an open-range zoo, Orana Wildlife Park, Christchurch, New Zealand. The kea is an endangered parrot (Robertson et al. 2013), that inhabits the mountains and rainforests of New Zealand's South Island. They are omnivorous, with a diverse, predominantly plant-based diet (Greer et al. in press). Feathers were collected during a one week period in February, 2013 from an aviary

housing five adults. The zookeepers provided a diet sheet detailing the types and quantities of foods fed to the birds per day, along with a sample of listed food items. However, initially an incomplete list was supplied and, although subsequently updated, we did not receive samples of all the food items fed to the kea. From a total of 33 food items, 9 were missing so we substituted available literature values, covering 98% of the keas' diet. The raw data for each food item are presented in Table S1 (Supplementary Material). Listed food quantities were mostly in grams (wet weights) but where a subjective quantity was used (e.g., one large banana), an example of the item was weighed to the nearest gram. Wet:dry weights were obtained by drying a sample of each food to constant mass in a 60°C oven. We used the dry weights of food fed per day in all calculations. Zookeepers were consulted to establish kea food preferences and each food was marked as 'preferred' or 'non-preferred'. Zoo-keeping protocols require all types of food to be spread throughout the enclosure to prevent monopolisation of resources by dominant individuals. All birds were held on this diet for more than six months prior to sample collection, during which time a moult had occurred.

Isotope values are reported in parts per thousand (‰) and standard definitions for isotopic compositions (δ^{13}C and δ^{15}N) are used throughout (see Coplen 2011).

Δ^{13}C and Δ^{15}N values were calculated as follows:

Firstly, the proportion (p) of each food item (m) in the total daily diet was calculated as

$$p_m = \frac{\text{dry weight}_m}{\text{dry weight}_{total}} \qquad \text{(Equation 1)}$$

However, foods can vary widely in their per cent carbon or nitrogen concentrations (%C or %N, respectively). Unless elemental concentration is accounted for, foods with high %C or %N can exert an undue influence on the calculated discrimination factor (Phillips & Koch 2002). Therefore, we

incorporated %C and %N into our equations to account for the differing elemental concentrations of every food item (following Martínez del Rio & Wolf 2005). The contribution (E) made to the total pool of element *X* by each food item was calculated as

$$EX_{\mathrm{m}} = \frac{\%X_{\mathrm{m}}}{\%X_{\mathrm{total}}} \qquad \text{(Equation 2)}$$

Then dietary δ^{13}C and δ^{15}N values (weighed by elemental concentration) were calculated using mass balance as

$$\delta^{y}X_{\mathrm{diet}} = \sum_{\mathrm{m}=1}^{\mathrm{n}} \delta^{y}X_{\mathrm{m}} \left(\frac{\mathrm{p_m} EX_{\mathrm{m}}}{\sum_{\mathrm{m}=1}^{\mathrm{n}} (\mathrm{p_m} EX_{\mathrm{m}})} \right) \qquad \text{(Equation 3)}$$

where, n is the number of food items.

Finally, discrimination factors were calculated as

$$\Delta^{y}X = \mathrm{mean}\ \delta^{y}X_{\mathrm{feather}} - \delta^{y}X_{\mathrm{diet}} \qquad \text{(Equation 4)}$$

In order to investigate the potential impact of metabolic routing we also calculated stable carbon and nitrogen discrimination factors assuming 100% metabolic routing of protein to tissue. The contribution of each food item to the pool of total dietary protein (O) was calculated as:

$$O_m = \frac{\%O_m}{\%O_{total}} \quad \text{(Equation 5)}$$

New dietary $\delta^{13}C$ and $\delta^{15}N$ values which assumed complete routing of dietary protein to tissue synthesis were then calculated using mass balance as:

$$\delta^y X_{routing} = \sum_{m=1}^{n} \delta^y X_m \left(\frac{p_m E X_m O_m}{\sum_{m=1}^{n} (p_m E X_m O_m)} \right) \quad \text{(Equation 6)}$$

To account for any potential impact from kea ingesting additional plants and animals from within their enclosure, we also modelled a scenario where kea incorporated 10% alpine plants and 10% invertebrates into their diet (plants: n = 84; $\delta^{13}C$ = -28.92‰ ± 2.39 and $\delta^{15}N$ = -4.56‰ ± 3.68 and invertebrates: n = 19; $\delta^{13}C$ = -25.76‰ ± 3.16; $\delta^{15}N$ = 2.13‰ ± 3.10; raw data available in Table S2). In addition, we modelled kea eating only the more preferred dietary items, because our data were based on the quantity of foods provisioned as opposed to ingested. These two conditions represent extreme limits to the discrimination factors as consultations with zookeepers indicated that kea devoted very little time to sourcing their own food within the enclosure and that most food was eaten at each feed. Final discrimination factors were calculated using the no routing and full routing of protein conditions only. Means are reported ± standard deviations throughout. All calculations were carried out in Microsoft Excel 2010 (Microsoft Corporation, Washington, USA).

Differences between the $\delta^{13}C$ and $\delta^{15}N$ values from kea blood and feathers

Blood and feather samples were collected simultaneously from 19 wild kea nestlings (seven females and 12 males from 14 different nests) in the final stages of fledgling feather growth, in Oct –

Dec, 2011 and Oct, 2012. All nestlings were sourced from nests located in Westland National Park on the South Island of New Zealand (43°13'S, 170°10'E). Kea incubate their eggs for 22-24 days and chicks remain in the nest for a further three months before fledging (Kemp 2013). Egg yolk provides a source of nutrients to birds for the first few days after hatching (Moran 2007) and could influence the δ^{13}C and δ^{15}N values of tissues synthesised during that period. However, as the turnover of blood for medium-sized birds is estimated at c. six weeks (American crow *Corvus bruchyrhynchos*; Hobson & Clarke 1993) and primary feather growth begins only three to four weeks prior to fledging (Renton 2002), yolk δ^{13}C and δ^{15}N values are an unlikely source of bias here.

The top 2 cm of a 1st primary (P1) and 10th primary (P10) feather of each bird were clipped and stored in a sealed plastic bag until processing. Approximately 0.3 cc of blood was drawn from the brachial wing vein of each bird and a few drops were stored in 70% ethanol for later analysis. Storage in 70% ethanol does not affect tissue δ^{15}N values and is thought to have a negligible effect on δ^{13}C values (Halley et al. 2008); however, Bugoni et al. (2008) found a significant effect on the δ^{13}C value of blood stored in absolute ethanol. To ensure that no preservation artefacts were introduced by the long-term storage of blood in 70% ethanol, we also took blood samples from nine fledgling kea outside Arthur's Pass Village (42°54'S, 171°34'E) and split each sample between 70% ethanol and an empty vial which was frozen at -20°C for up to one week and then stored at -80°C until analysis (always ≤9 months).

We averaged the δ^{13}C and δ^{15}N values from the P1 and P10 feathers to give a single feather for each nestling. Pairwise t-tests were then used to determine if blood and feather δ^{13}C and δ^{15}N values differed from one another and if the method of blood sample storage affected stable isotope values. We used linear regressions to test if δ^{13}C or δ^{15}N values from blood could be reliably used to predict feather values. Tissue $\Delta^{y}X$ value differences were calculated by $\Delta^{y}X_{\text{feather}} - \Delta^{y}X_{\text{blood}}$. Means are reported ± 1 standard deviation throughout. All analyses were conducted using SPSS Statistics 21 (IBM Corporation, NY 10589, USA).

Exclusions

We lost data from both of one nestling's feather samples and from one frozen blood sample due to a mass spectrometer technical malfunction so we excluded all data from these birds from statistical analyses. One nestling (J2) had a higher $\delta^{15}N_{blood}$ than $\delta^{15}N_{feather}$ value (Table S3). This is the opposite pattern to that found in all constant diet studies (except Hobson & Clarke 1992), therefore we conclude that J2's pattern is anomalous and indicates a change in diet at a crucial time. The slower metabolic turnover of blood (c. six weeks) than fledgling feather growth (c. three weeks) means that a substantial shift from higher to lower trophic level in the diet fed to J2 between the start of blood synthesis and feather growth could result in its feathers having a lower $\delta^{15}N$ value than its blood. J2 also had the largest tissue difference in $\delta^{13}C$ (3.30‰), further suggesting a change in diet. Consequently, in order to increase the applicability of our regression equations, we excluded data from J2. However, this case highlights the need for caution when applying this methodology, particularly in opportunistic species which may take advantage of temporary bonanzas (e.g., increases in invertebrate numbers or occasional vertebrate carcasses) to supplement their diet and that of their young.

Sample Preparation

Feathers soaked in 2:1 chloroform:methanol solution for 24 h were rinsed twice in fresh solution and air dried in a fume cupboard for 48 h. The top 1 cm of the inner feather vane was removed and finely clipped. Blood and food samples were dried to constant mass in a 60°C oven and ground to talc powder consistency in a ball mill (Retsch MM2000, Hahn, Germany). Samples were homogenised and weighed out on an ultra-microbalance (accurate to 0.1 µg; Mettler-Toledo UMX2, Greifensee, Switzerland) to 0.5 - 0.7 mg for kea tissue and 3.5 - 5 mg for food and inserted into individual 4 x 6 mm tin capsules for mass spectrometer analysis. In three cases, the food type was a

mixture (e.g., insectivore mix or seed mix), so we prepared repeated samples. We did not extract lipids because recent work suggests that carbohydrates and lipids are also used for tissue synthesis and thus a portion of lipid in the diet is desirable to provide the full suite of dietary macro-molecules (Newsome et al. 2014). Data on the protein content of each food item were sourced from the US National Nutrient Database (2014).

Mass Spectrometry

Samples were analysed for $\delta^{13}C$, $\delta^{15}N$, %C and %N using a Costech Elemental Combustion System (ECS) 4010 (Costech Analytical Technologies, California, USA) connected to a Delta V Plus Isotope Ratio Mass Spectrometer (IRMS; Thermo Fischer Scientific, Massachusetts, USA) via a Finnigan Conflo III (Thermo Fischer Scientific, Massachusetts, USA). All samples were loaded into a ZeroBlank autosampler with an isolation valve (Costech Analytical Technologies, California, USA) and were individually combusted at 1050°C under a continuous flow (c. 110 ml/min) of ultra-high purity helium (>99.99%). Molecular N_2 and CO_2 were separated using a gas chromatography column housed in the ECS and held at a static 45°C. IRMS fast peak jumps were calibrated at least daily, and reference gas linearity tests were performed at the start of every other analytical sequence. Internal precision (the standard deviation across ten reference gas analyses i.e., zero-enrichment test) was determined prior to every analytical sequence and was always <±0.06‰ for both $\delta^{13}C$ and $\delta^{15}N$. Data were normalised to Vienna PeeDee Belemnite for $\delta^{13}C$ and Air for $\delta^{15}N$ using a stretch-and-shift 2-point normalisation based on replicate analyses of certified reference materials within individual analytical sequences. External precision (the standard deviation of replicate analyses of certified reference materials and internal lab-check standards over the course of the study) was <±0.20‰ for both $\delta^{13}C$ and $\delta^{15}N$.

Results

Determination of discrimination factors for feathers

The food items which comprised the diet of kea at the zoo varied widely in their $\delta^{13}C$ (mean = -24.83‰ ± 6.66; range = -33.20‰ to -11.15‰) and $\delta^{15}N$ values (mean = 3.97‰ ± 3.18; range = -33.20‰ to -11.15‰); their %N (mean = 1.09% ± 7.95; range = 0.17% to 9.40%); and their protein content (mean = 6.15% ± 10.20; range = 0.30% to 52.00%; Table S1). In contrast, the kea feathers collected from the enclosure varied little in either their $\delta^{13}C$ (mean = -23.28‰ ± 0.36; range = -23.75‰ to -22.5‰) or $\delta^{15}N$ (mean = 7.32‰ ± 0.45; range = 6.63‰ to 8.23‰) values. The raw data for these 18 feathers are available in Table S4.

The $\Delta^{13}C_{diet\text{-}feather}$ values were consistently c. 4‰ regardless of the model conditions, ranging by only ≤0.30‰ (Table 1). The $\Delta^{15}N_{diet\text{-}feather}$ values varied somewhat more widely in the extreme conditions (2.78‰ to 4.00‰; Table 1). However, when we based our calculations on the full diet provided to kea there was a variation of just 0.40‰ (No routing: $\Delta^{15}N_{diet\text{-}feather}$ = 2.90‰; complete routing: $\Delta^{15}N_{diet\text{-}feather}$ = 3.30‰). We suggest that future stable isotope studies on kea adopt $\Delta^{13}C_{diet\text{-}feather}$ = 4.00‰ ± 0.03; $\Delta^{15}N_{diet\text{-}feather}$ = 3.10‰ ± 0.20.

Differences between the $\delta^{13}C$ and $\delta^{15}N$ values from kea blood and feathers

Across kea nestlings, the stable carbon and nitrogen isotopic compositions were widely disparate, ranging by c. 7‰ for $\delta^{13}C$ and $\delta^{15}N$ within-tissue values (Table 2), indicating different dietary sources. Within individuals, feathers were significantly higher than blood for both their stable carbon ($\delta^{13}C_{feather}$ = -21.08‰ ± 1.99; $\delta^{13}C_{blood}$ = -22.97 ± 1.93; t_{17} = 9.90, p <0.001) and nitrogen ($\delta^{15}N_{feather}$ = 2.92‰ ± 1.71; $\delta^{15}N_{blood}$ = 2.11‰ ± 1.87; t_{17} = 7.62, p <0.001) isotope compositions.

Feathers and blood were also significantly different in their C:N ratios (t_{17} = 5.35, p <0.001; Table 2). Individual nestling blood and feather stable isotope values and C:N ratios are available in Table S3. There were no differences between frozen blood samples and those stored in 70% ethanol in their δ^{13}C (t_7 = 0.24, p = 0.816; mean difference = 0.02‰ ± 0.19) or δ^{15}N values (t_7 = -2.18, p = 0.066; mean difference = -0.44‰ ± 0.58).

Regression equations

We calculated linear regression equations to predict $\delta^{13}C_{feather}$ from $\delta^{13}C_{blood}$ values; and $\delta^{15}N_{feather}$ from $\delta^{15}N_{blood}$ values. The values from feathers and blood were positively related and both regression equations explain over 90% of the variance ($\delta^{13}C_{feather}$ = 0.97 (±0.29) $\delta^{13}C_{blood}$ + 1.13 (±6.61), R^2 = 0.93, $F_{1,15}$ = 61.69, p <0.001; $\delta^{15}N_{feather}$ = 0.92 (±0.19) $\delta^{15}N_{blood}$ + 1.04 (±0.52), R^2 = 0.97, $F_{1,15}$ = 417.84, p <0.001; Fig. 1). The difference between actual and predicted feather values for δ^{13}C was small (<0.50‰) in 13 cases, moderate (<1.00‰) in three cases, with one large (>1.00%) difference. For δ^{15}N there was a small difference in 15 cases, and a moderate difference in two cases. The slope of our regression equation for predicting kea $\delta^{13}C_{feather}$ from $\delta^{13}C_{blood}$ values was not significantly different from that of the corresponding equation calculated for use across marine birds (t_{48} = 0.01, p = 0.989; $\delta^{13}C_{marine\text{-}bird\ feather}$ = 0.97 (±0.02) $\delta^{13}C_{marine\text{-}bird\ blood}$ + 0.96 (±0.41); Cherel et al. 2014; Fig. 1); however the slopes of the regression equations derived to predict kea and marine bird $\delta^{15}N_{feather}$ from $\delta^{15}N_{blood}$ values were significantly different (t_{48} = 2.05, p = 0.046; $\delta^{15}N_{marine\text{-}bird\ feather}$ = 1.01 (±0.06) $\delta^{15}N_{marine\text{-}bird\ blood}$ + 0.45 (±0.67); Cherel et al. 2014; Fig. 1).

Discussion

Here we report the first diet-to-tissue discrimination factors for a parrot; and the first equations for predicting feather carbon and nitrogen stable isotope values from blood values to be determined

using wild birds. When studying species or tissues for which discrimination factors are as yet unknown, the application of these methodologies has the potential to greatly improve the accuracy of dietary mixing models outputs and trophic levels comparisons.

Determination of discrimination factors for feathers

The $\Delta^{13}C$ values we obtained for kea feathers were highly consistent (c. 4‰), varying ≤0.30‰ even under the most extreme model conditions. A stable carbon isotope discrimination factor of 4‰ falls within the higher range of reported values (Caut et al. 2009; Cherel et al. 2014), which can, in part, be explained by the choice of tissue. Feathers typically have more positive $\delta^{13}C$ and $\delta^{15}N$ values than blood or muscle (Caut et al. 2009) and converting $\delta^{13}C_{feather}$ to $\delta^{13}C_{blood}$ values using the kea-specific regression equation calculated here reduces $\Delta^{13}C$ to 2.96‰. However, this value still exceeds the commonly assumed 0 - 1‰ value (Post 2002), further emphasising the need for species-specific discrimination factors to be determined before incorporating them into highly-sensitive Bayesian mixing models (Bond & Diamond 2011). $\Delta^{15}N_{diet\text{-}feather}$ values differed by 1.16‰, the highest and lowest values being obtained when we assumed that kea took 20% of their diet from plants and animals within their enclosure or ate only preferred foods. However, both of these model conditions represent extreme limits and $\Delta^{15}N_{diet\text{-}feather}$ values from the complete diet fed to kea varied by only 0.41‰. The stable nitrogen isotope discrimination factor of 3.10‰ obtained here for kea is close to the frequently adopted value of 3.40‰ per trophic level (Post 2002).

The unusually high stable carbon isotope discrimination factor reported here may be the consequence of the kea's extremely high basal metabolic rate (BMR; 37% higher than expected from their body mass; McNab & Salisbury 1995). Given that respiration and BMR are positively related (Mansell & MacDonald 1990), this suggests that kea also have a higher rate of CO_2 production. The $\delta^{13}C$ value of an animal's exhaled breath is typically lower than its dietary $\delta^{13}C$ value; therefore

respiration is a likely cause of diet-to-tissue ^{13}C enrichment and a physiologically similar animal which respires more should have an increased stable carbon isotope discrimination factor (DeNiro & Epstein 1978). Published $\delta^{13}C_{feather}$ values are only available for two other parrot species (red-crowned parakeet *Cyanoramphus novaezelandiae,* Hawke & Holdaway 2009; cape parrot *Poicephalus robustus*; Symes & Woodborne 2009). In both studies, the parrots were found to have unexpectedly positive $\delta^{13}C_{feather}$ values when compared to the surrounding vegetation. It is noteworthy that the BMR of the red-crowned parakeet is also elevated, at 112% of the expected rate (McNab & Salisbury 1995) and it has been suggested that parrots generally may have higher BMRs than other species (McNab 2012), although Montgomery et al. (2012) found no evidence of this when comparing parrots and quails (Order: Galliformes). Future research targeting parrot $\Delta^{13}C_{diet\text{-}tissue}$ values, and their comparison to discrimination factors determined for other birds across a range of BMRs and metabolic pathways is urgently needed.

Differences between the $\delta^{13}C$ and $\delta^{15}N$ values from kea blood and feathers

$\delta^{13}C$ and $\delta^{15}N$ values from kea feathers were significantly higher than those from their blood. With the exception of $\delta^{15}N$ for one nestling, these differences were highly consistent, allowing the derivation of regression equations to confidently predict feather $\delta^{13}C$ and $\delta^{15}N$ values from blood values within kea.

Our equation for $\delta^{13}C$ was remarkably similar to one derived for use across marine bird taxa (Cherel et al. 2014), suggesting that $\delta^{13}C$ differences between feathers and blood may be highly consistent across bird taxa in general. The differences between $\delta^{13}C$ values from blood and feathers are likely the result of the tissues' differing biochemical compositions (Wolf & Martínez del Rio 2009). Lipid $\delta^{13}C$ values are lower than protein values and lipids are present in greater quantities in kea blood than feathers (verified here by blood's significantly higher C:N values – an inversely-

related, proxy for lipid content; Post et al. 2007). Different amino acids also vary in their isotopic ratios so the differing amino acid composition of these tissues could also contribute to these $\delta^{13}C$ differences (Wolf & Martínez del Rio 2009; Cherel et al. 2014), seemingly to the same degree in kea as for marine birds.

Applying the marine bird equation for predicting $\delta^{15}N_{feather}$ from $\delta^{15}N_{blood}$ values (Cherel et al. 2014) to kea tissues was less successful, resulting in mostly (76%) moderate or large errors and the consistent underestimation of $\delta^{15}N_{feather}$ values at lower levels of $\delta^{15}N_{blood}$ (<2‰). This may be due to a difference between kea and marine birds in their metabolic routing of protein. Feather production relies particularly heavily on cysteine, a semi-essential sulphur amino acid which can be only ingested directly from food or synthesised from the essential amino acid methionine (Murphy 1990). Both cysteine and methionine are present in greater quantities in high protein foods. Omnivorous birds, such as kea, may not obtain enough cysteine or methionine for feather production from the plant portion of their diet, and may route most animal protein consumed into feather production rather than into the creation of other tissues. On the other hand, predominantly carnivorous birds i.e., marine birds, may not need to route animal protein specifically for feather production. Increases in dietary animal protein content has been experimentally demonstrated to be positively related to $\delta^{15}N$ (Pearson et al. 2003), so if more animal protein is being routed to feather production over blood in kea but not in marine birds, this would explain why the marine bird equation (Cherel et al. 2014) underestimated kea $\delta^{15}N_{feather}$ values. However, there is evidence to suggest that a contrary effect occurs, whereby increases in protein *quality* lead to a reduction in $\delta^{15}N$ and a lively debate surrounds this issue (e.g., Caut et al. 2009; Perga & Grey 2010; Kurle et al. 2013).

An alternative explanation is that because kea obtain less cysteine from their diet than marine birds do, they have to synthesise more of it from methionine. This would increase the number of metabolic transanimations, the isotopic fractionations involved would increase the $\delta^{15}N$ of the product amino acid (Chikaraishi et al. 2009), and consequently the $\delta^{15}N$ of the feathers,

leading to the observed trend. We note, however, that little is known about the isotopic composition of cysteine and methionine in feathers, as they were not measured in the only published study on feather amino acid isotopes (Lorrain et al. 2009). We propose that the degree of difference between blood and feather δ^{15}N values may be more closely related to the bird's degree of carnivory than the ecosystem (marine or terrestrial) they inhabit.

Conclusion

These methodologies have considerable potential to improve upon the large-scale adoption of discrimination values from review articles, especially considering that these may be based on different tissues or sometimes vastly different animal taxa. Indeed, a database of discrimination factors for many species across orders may reveal trends that are not yet visible through the current paucity of data. Although we conducted this work using an avian species, these methodologies can be easily adapted to other classes, including mammals and reptiles. To determine $\Delta_{\text{diet-tissue}}$ values directly only the tissue selected must be changed. To convert between tissues, shaved mammalian hair can be used instead of feathers, with the length of hair selected matched to the amount grown during the synthesis of blood, or for reptiles, new scales grown in preparation for moulting can be sampled and compared with blood (depending on tissue turnover rates). We hope that researchers will apply these methodologies before relying on generic discrimination factors or making cross-tissue comparisons.

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Data Accessibility

The following have been uploaded as online supporting information:

- Captive kea diet (Table S1)

- Alpine plant and invertebrate isotope ratios (Table S2)

- Wild kea nestling feather and blood isotope ratios (Table S3)

- Captive kea feather isotope ratios (Table S4)

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Table 1: $\delta^{13}C_{diet}$ and $\delta^{15}N_{diet}$, and $\Delta^{13}C_{diet\text{-}feather}$ and $\Delta^{15}N_{diet\text{-}feather}$ values calculated for captive kea for each model condition: no routing of protein, complete routing of protein, the inclusion of 10% plant and 10% invertebrates in their diet, and the ingestion of preferred foods only. All models take the elemental concentration of food items into account. $\Delta^{13}C$ and $\Delta^{15}N$ values were calculated using $\delta^{13}C_{feather}$ = -23.28‰ and $\delta^{15}N_{feather}$ = 7.32‰.

	Diet $\delta^{13}C$ (‰)	Diet $\delta^{15}N$ (‰)	$\Delta^{13}C$ (‰)	$\Delta^{15}N$ (‰)
No routing of protein	-27.25	4.42	3.97	2.90
Complete routing of protein	-27.30	4.01	4.02	3.30
10% plant, 10% invertebrate	-27.27	3.37	3.98	4.00
Preferred foods only	-27.00	4.53	3.72	2.78

Table 2. Mean, standard deviation (SD) and range of $\delta^{13}C$, $\delta^{15}N$ and C:N values for 1st Primary (P1) and 10th Primary (P10) feathers, and blood sampled simultaneously from wild-caught kea nestlings (*n* = 18).

	$\delta^{13}C$ (‰)		$\delta^{15}N$ (‰)		C:N	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
P1 Feather	-21.03 ± 2.04	-26.48 to -19.19	2.88 ± 1.75	0.84 to 7.74	3.22 ± 0.08	3.07 to 3.36
P10 Feather	-21.14 ± 1.94	-26.14 to -19.22	2.96 ± 1.67	1.25 to 7.51	3.30 ± 0.10	3.09 to 3.52
Blood	-22.97 ± 1.93	-27.95 to -20.62	2.11 ± 1.87	0.30 to 7.03	3.36 ± 0.05	3.24 to 3.45
Feather*	-21.08 ± 1.99	-26.31 to -19.31	2.92 ± 1.71	1.04 to 7.63	3.26 ± 0.07	3.08 to 3.39

*denotes a single value for feather obtained by averaging the P1 and P10 values for each nestling.

Figures

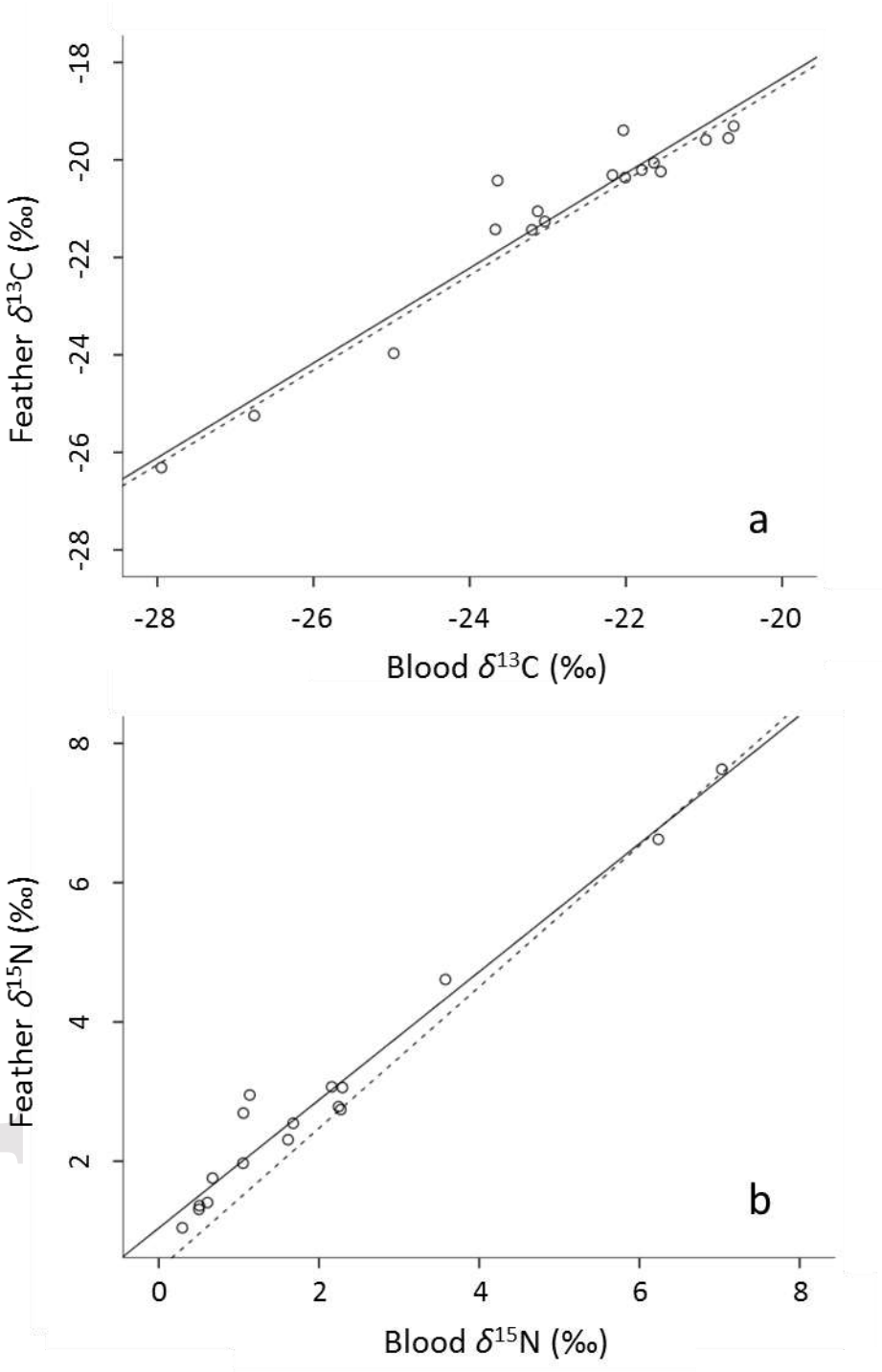

Figure 1. The relationships between the δ^{13}C (a) and δ^{15}N (b) values from parrot blood and feathers. Open circles represent individual kea nestlings. Solid lines indicate the regression equations calculated here; dashed lines indicate the corresponding equations derived for marine birds (Cherel et al. 2014).