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Dietary protein influences the rate of ^{15}N incorporation in blood cells and plasma of Yellow-vented bulbuls (*Pycnonotus xanthopygos*)

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SUMMARY

The rate at which an animal's tissues incorporate the isotopic composition of food determines the time window during which ecologists can discern diet changes. We investigated the effect of protein content in the diet on the incorporation rate of ^{15}N into the plasma proteins and blood cells of Yellow-vented bulbuls (*Pycnonotus xanthopygos*). Using model comparison analyses, we found that one-compartment models described incorporation data better than two-compartment models. Dietary protein content had a significant effect on the residence time of ^{15}N in plasma proteins and blood cells. The diet with the highest protein content led to a ^{15}N retention time of 21 and 5 days for cells and plasma, respectively. In contrast, average ^{15}N retention time in the cells and plasma of birds fed on the diet with the lowest protein was 31 and 7 days, respectively. The isotopic discrimination factor $\Delta^{15}N=\delta^{15}N_{tissues}-\delta^{15}N_{diet}$ was also dependent on dietary protein content, and was lowest in birds fed the diet with the highest protein content. Blood, plasma and excreta were enriched in ^{15}N relative to diet. In contrast, ureteral urine was either significantly depleted of ^{15}N in birds fed the diet with the lowest protein content or did not differ in $\delta^{15}N$ from the diets with the intermediate and high protein content. Thus, isotopic incorporation rates and tissue-to-diet discrimination factors cannot be considered fixed, as they depend on diet composition.

Key words: incorporation rate, stable isotopes, Yellow-vented bulbul, protein intake, diet reconstruction.

INTRODUCTION

The measurement of naturally occurring carbon and nitrogen stable isotopes in animal tissues is a powerful tool in the study of dietary ecology. The isotopic composition of an animal reflects the isotopic composition of its diet in a predictable manner (DeNiro and Epstein, 1978; DeNiro and Epstein, 1981). However, after a diet change, the isotopic composition of an animal's tissues does not change instantaneously [Cerling et al. (Cerling et al., 2007) and references therein]. Different tissues incorporate the diet's isotopic composition at different rates (Tieszen et al., 1983). The differences in isotopic incorporation among tissues can be useful as tissues with fast incorporation rates reveal short time scale changes in diet, whereas tissues with slow incorporation rates integrate the isotopic composition of the diet over longer intervals (Pearson et al., 2003; Podlesak et al., 2005).

Tieszen et al. (Tieszen et al., 1983) hypothesized that tissues with high metabolic activity would also have high rates of isotopic incorporation. The notion of the existing relationship between animal basal metabolic rate (BMR) and its isotopic fractional turnover rate was challenged by Voigt et al. (Voigt et al., 2003) and Voigt and Matt (Voigt and Matt, 2004). They studied carbon and nitrogen turnover rates in blood and wing membrane of two nectarivorous bat species (*Leptonycteris curasoae* and *Glossophaga soricina*), which have high mass-specific BMRs. Hence, it was expected that the isotopic fractional turnover rate of these species would also be high. Surprisingly, the turnover rates of both elements were found to be the lowest measured so far in a vertebrate. This study implies that N isotopic turnover rate might be unrelated to the animal's BMR as was commonly thought. However, the diets used in Voigt and colleagues' (Voigt et al., 2003) experiments had protein contents that were insufficient to meet the bat's requirements (Herrera et al., 2006), and the bats lost mass. When Mirón et al. (Mirón et al., 2006) fed Pallas' long-tongued bats (*G. soricina*) diets with adequate protein levels, they had much higher levels of isotopic incorporation than those reported by Voigt et al. (Voigt et al., 2003). The contrast between the results of Voigt et al. (Voigt et al., 2003) and Mirón et al. (Mirón et al., 2006) suggests that the dietary protein level is a controlling factor in the rate of isotopic incorporation.

The idea of a relationship between metabolic rate and both C and N isotopic incorporation rates was expanded upon by Carleton and Martínez del Rio (Carleton and Martínez del Rio, 2005) who interpreted 'high metabolic activity' as high rates of protein synthesis and catabolism. They tested the hypothesis that chronic cold exposure, and hence an increase in metabolic rate, would increase the isotopic incorporation rate of ^{13}C and ^{15}N in House sparrow (*Passer domesticus*) red blood cells. They found that despite an increased metabolic rate, cold exposure had no effect on ^{15}N incorporation rate, and had only a small effect on ^{13}C incorporation rate. They concluded that the relationship between metabolic rate and the rate of isotopic incorporation into an animal's tissue is indirect and probably mediated by protein turnover rate. Dietary proteins are known to have a regulatory effect on protein synthesis and degradation (Millward, 1989; Lobley, 2003). Physiologists have documented increases in protein synthesis resulting from increased protein intake in fish (Millward, 1989; Houlihan et al., 1995), domestic chickens (Dror et al., 1997) and mammals (Yahya et al., 1994; Wessels et al., 1997; Williams

Table 1. Experimental diet composition

Ingredients (g)	Soy diet	Casein – low	Casein – medium	Casein – high
Soy protein	6	–	–	–
Casein protein	–	4.8	9.6	19.2
Sodium chloride	1	1	1	1
Calcium phosphate	1	1	1	1
Vitamins and minerals	1	1	1	1
Soy oil	4	4	4	4
Banana	400	400	395	387
Water	580	580	580	580
Agar	6	6	6	6

The amounts shown are for 1 day for 10 birds. These amounts were adapted to the number of birds consuming the diet for 3 days, while keeping the same proportion of ingredients as specified in the table.

et al., 2001), including humans (Foulliet et al., 2001). Hence, we should expect to find a correlation between protein intake and N fractional isotopic turnover rate.

We conducted a feeding experiment on captive Yellow-vented bulbul (*Pycnonotus xanthopygos*), a frugivorous bird of the Old World, to test the conjecture that dietary protein influences the rate of isotopic incorporation. Birds received diets that had similar caloric values and similar $\delta^{15}N$ isotopic signatures but varied in their protein content. Hence, we could isolate the effect of dietary elemental concentrations on fractional N isotopic turnover rate in tissues. The goals of the experiment were to determine the effect of protein intake on: (1) nitrogen fractional turnover rate of red blood cells and plasma, and (2) $\delta^{15}N$ tissue–diet discrimination factors of blood cells, plasma, excreta and ureteral urine. We hypothesized that the N fractional isotopic turnover rate and the $\delta^{15}N$ tissue–diet discrimination factors would increase with increasing protein intake. The depletion in ^{15}N in nitrogenated excreted products has often been invoked as the cause of the tissue-to-diet enrichment in ^{15}N (Minagawa and Wada, 1984; Martínez del Rio and Wolf, 2005). Thus, we also hypothesized that both ureteral urine and excreta would be depleted of ^{15}N relative to diet.

Isotopic incorporation data are commonly analysed using simple, one-compartment models, with first-order kinetics (Carleton and Martínez del Rio, 2005; Mirón et al., 2006; Cerling et al., 2007). Cerling et al. (Cerling et al., 2007) challenged the use of these models and championed the use of more complex multi-compartment models. We used our data and information theoretic model comparison methods to evaluate Cerling and colleagues' (Cerling et al., 2007) claim (Stephens et al., 2007). We used Akaike's information theoretic criteria to assess whether evidence supported the use of one-compartment or two-compartment models.

MATERIALS AND METHODS

Bird capture, care and maintenance

Thirteen Yellow-vented bulbuls *Pycnonotus xanthopygos* (Pycnonotidae; Hemprich and Ehrenberg 1833), mean body mass (M_b) 36.5±0.6 g, were used in the experiments. Yellow-vented bulbuls are not sexually dimorphic and we did not determine the sex of the individuals in our experiment. Birds were mist-netted 1 month before the experiment in Sde-Yaakov, northern Israel (under license from the Israel Nature and National Parks Authority). Birds were housed in individual cages (110 cm × 60 cm × 100 cm), in a room maintained at 25±2°C with a photoperiod of 12 h:12 h light:dark. The bottom of the cages was covered with paper that was changed daily. All birds received a standard maintenance banana-mash diet [based on Denslow et al. (Denslow et al., 1987)], with soy protein as a protein source (soy diet, Table 1) for 75 days. To prevent a difference in the isotopic signature of the diet during that time, we used the same batch of bananas. All bananas were mixed using a blender, divided into portions and frozen. Every 3 days, one bag was thawed and the rest of the experimental ingredients were added. We used the same batch of each ingredient throughout the experiment; each was mixed prior to the experiments so that the diet throughout the experiment would have the same isotopic signature (casein protein, sodium chloride and calcium phosphate, Sigma Chemical Co., St Louis, MO, USA; soy protein–ARDEX® F dispersible isolated soy protein 066-921, Archer Daniels Midland Co., IL, USA; vitamin mix Omni-vit, Orlux, Belgium). The birds received 100 g of wet food every day and water was provided *ad libitum*.

Diet shift experiment

After consuming the first diet for 75 days, birds were divided randomly into three groups; each bird received a diet containing a different amount of casein protein (5 birds – low protein, 4 birds – medium protein, 4 birds – high protein; Table 1). Our diets fully satisfied the bird's nitrogen requirements (Tsahar et al., 2005). Again, we used the same batch of each ingredient during the experiment; each was mixed prior the experiments so that the diet throughout the experiment would have the same isotopic signature. The birds received the second diet for 95 days. Blood and excreta samples were taken on the day prior to the dietary switch (day 0) and then on days 2, 4, 9, 21, 35, 57, 80 and 95 of consuming the casein diet. Blood was collected (~100 μl) in heparinized microhaematocrit tubes by puncturing the brachial vein with a 28 gauge needle. Plasma was separated from cells after centrifugation (micro-haematocrit centrifuge model CL A4922X-1, International Equipment Co., Needham Heights, MA, USA) for 3 min. Ureteral urine samples were collected on day 95 by briefly inserting a closed-ended perforated cannula (Goldstein and Braun, 1989), custom-made of polyethylene tubing (PE200), into the bird's cloaca. On the same day, we also collected excreta samples for 24 h. Body mass was measured every sampling day. Both excreta and ureteral urine were collected and stored in a dilute (0.001 N) HCl solution. Birds were released at the capture site upon completion of the experiment. Samples were kept frozen (–20°C) until the end of the experiment. Plasma and blood cells were spread on glass slides and oven dried for 3–4 days at 50°C. All dried samples were scraped from the glass using a razor blade, and homogenized using a mortar and pestle.

All samples were ground into a fine powder before being loaded (30–70 μg) into tin capsules. Isotope ratios of food were measured in a continuous flow isotope ratio mass spectrometer (Finnigan Delta+XP, University of Wyoming's Light Stable Isotope Facility) with samples combusted in a Costech elemental analyser. The precision of these analyses was ±0.2‰ for both isotopes. Our standards were peptone ($\delta^{15}N$=5.60‰, AIR, USGS40 8542) and glycine ($\delta^{15}N$=0.73‰, AIR, IAEAN2). We included standards in every run to correct raw values obtained from the mass spectrometer. Stable isotope ratios were expressed using standard delta notation ($\delta^{15}N$) in parts per million (‰) as:

$$\delta^{15}N = (R_{sample}/R_{standard}–1) \times 1000 ,$$

Table 2. Elemental and isotopic composition of the ingredients of the experimental diets

	Soy protein	Casein	Bananas	Soy diet	Low protein	Medium protein	High protein
N(%)	14.98	15.0	0.9	2.21	1.1±0.15	1.5±0.3	2.6±0.5
C(%)	49.2	50.7	39.8	46.181	40.6±0.3	39.9±2.6	42.5±3.7
$\delta^{15}N$	1.5	7.6	1.76	1.21	3.9±0.1	4.5±0.3	5.0±0.2

where R_{sample} and $R_{standard}$ are $^{15}N/^{14}N$ ratios of the sample and the reference, respectively. Samples were referenced against atmospheric nitrogen (AIR). Table 2 lists the isotopic composition of our diet's ingredients.

Statistical analyses

Preliminary diagnoses on the use of one- or two-compartment models were performed using Cerling and colleagues' (Cerling et al., 2007) reaction-progress approach. Briefly, we plotted $\ln(1-F)$, where $(1-F)=[\delta^{15}N(t)-\delta^{15}N(\infty)]/[\delta^{15}N(0)-\delta^{15}N(\infty)]$ against time and assessed visually whether a single line or more than one line was needed to describe the data. Isotopic incorporation data were then fitted using a non-linear fitting routine (JMP®, version 6.0, SAS Institute, Cary, NC, USA) to either one- or two-compartment models using the following equations, respectively:

$$\delta^{15}N(t) = \delta^{15}N(\infty) - [\delta^{15}N(\infty) - \delta^{15}N(0)]\exp - (1/\tau)\text{time} \quad (1)$$

and

$$\delta^{15}N(t) = \delta^{15}N(\infty) - [\delta^{15}N(\infty) - \delta^{15}N(0)] [p\exp-(1/\tau_1)\text{time} + (1-p)\exp-(1/\tau_2)\text{time}] , \quad (2)$$

where $\delta^{15}N(0)$ and $\delta^{15}N(\infty)$ represent the initial and asymptotic nitrogen isotopic compositions. Eqns 1 and 2 differ slightly from those used in most isotopic incorporation studies (Carleton and Martìnez del Rio, 2005; Mirón et al., 2006; Cerling et al., 2007) in their use of the reciprocal of the fractional incorporation rate ($\tau_i=1/k$, days) as a parameter to describe incorporation rate. We chose to use this parameter for two reasons: (1) it has a clear intuitive interpretation as the average retention (or residence) time of ^{15}N for the one-compartment model, and (2) the non-linear routine used in our analysis gave asymptotic s.e.m. estimates. In previous studies, such as those listed above, researchers estimated the fractional rate of incorporation ($k=1/\tau$) and used it to estimate the half-life of an element in a tissue ($t_{1/2}=\tau\times\ln(2)=\ln(2)/k$). This approach does not allow for estimates of uncertainty in the calculation of these half-lives. Our approach allows estimates of uncertainty in the calculation of the parameter that isotopic ecologists care about – how long does an element stay in a tissue? To assess the weight of evidence in favour of a one- or a two-compartment model, we compared the Akaike's information criteria corrected for small samples (AICc) of the two models and chose the model with the lowest AICc value (Burnham and Anderson, 2002). Burnham and Anderson (Burnham and Anderson, 2002) propose using the difference in AICc ($\Delta i=AICc_i-AICc_{min}$, where $AICc_{min}$ is the lowest value in a comparison) as a measure of the plausibility of an alternative model. They suggest that high values of Δi ($\Delta i>2$) indicate low support for the alternative model [see p. 70 in Burnham and Anderson (Burnham and Anderson, 2002)]. If AIC_c revealed that the weight of evidence supported a one-compartment model, we used τ as an estimate of average retention time, whereas if it supported a two-compartment model, we estimated average retention time as:

$$\tau_2 = p\tau_1 + (1-p)\tau_2 . \quad (3)$$

We estimated isotopic discrimination ($\Delta^{15}N$) as $\delta^{15}N(\infty)_{tissues}-\delta^{15}N_{diet}$. Finally, we examined the effect of protein content in the diet on average ^{15}N retention time and $\Delta^{15}N$ (defined as $\Delta^{15}N=\delta^{15}N_{tissues}-\delta^{15}N_{diet}$) with one-way analysis of

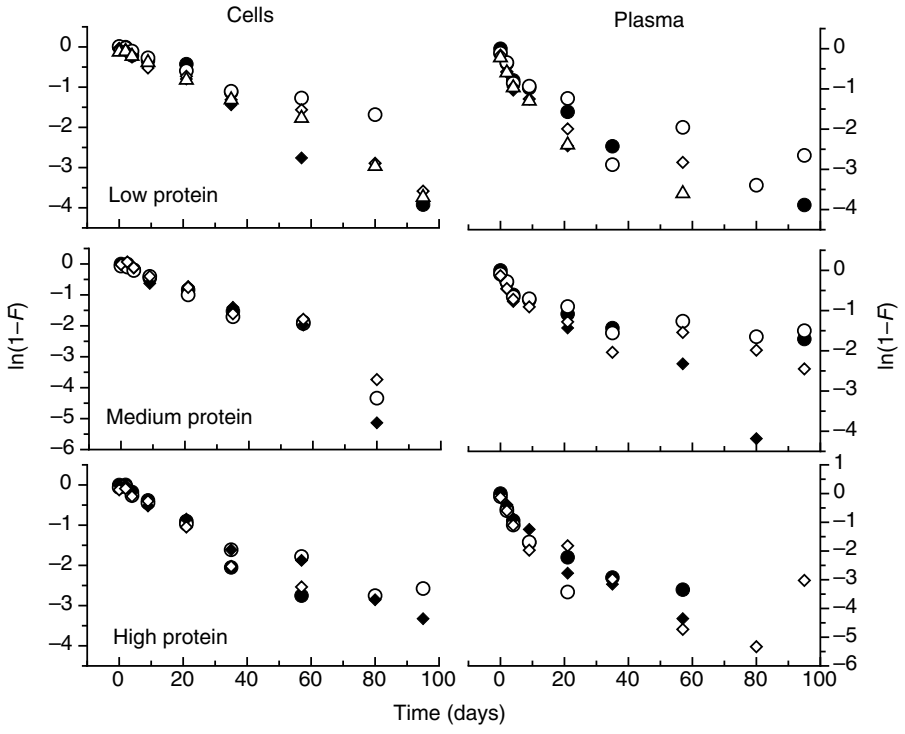

Fig. 1. The reaction progress variable plots all showed decreasing trends between ln(1–*F*) and time. However, it is difficult to discern from these plots whether a one- or a two-compartment model should be applied to each data set. Different symbols in each panel label data for each individual. For all figures, see List of abbreviations and symbols for definitions.

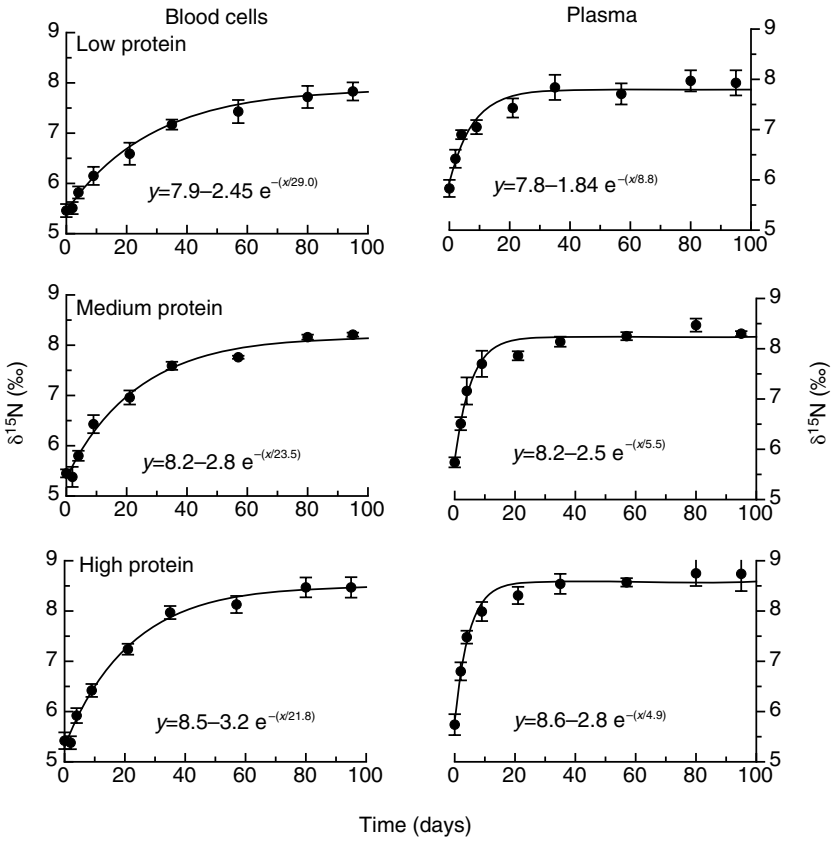

Fig. 2. One-compartment, first-order kinetic models of isotopic incorporation models adequately described the incorporation of ^{15}N into the blood cells and plasma of Yellow-vented bulbuls. Points are means and bars are s.e.m. The curves were fitted using mean values of $\delta^{15}N(\infty)$, $[\delta^{15}N(\infty)–\delta^{15}N(0)]$ and τ. The values for these parameters are shown in the equations. Both equations and fitted curves are presented for descriptive purposes only. All statistical analyses were conducted on data for individuals.

variance followed by Tukey–Kramer multiple comparisons among means ($P<0.05$). We used one-sample Student's *t*-tests to compare the $\delta^{15}N$ of plasma, cells, excreta and ureteral urine with that of diet.

RESULTS

The reaction progress variable plots are shown in Fig. 1 and demonstrate that at most two compartments are needed to describe the data. The method, however, does not allow clear determination of whether the data support a one- or two-compartment model. In all cases, AICc supported the one- over the two-compartment model. Δi values ranged from 2.1 to 15.8, suggesting little support for the two-compartment model. Consequently, we used τ to characterize the residence time of ^{15}N in plasma and blood cells. The one-compartment model was not only better than the two-compartment model but it also described the data adequately well ($r^2>0.94$; Fig. 2).

The protein content of the diet had a significant effect on the residence time of ^{15}N ($F_{2,10}=5.54$ and $F_{2,10}=4.73$, $P<0.05$, for cells and plasma, respectively; Fig. 3). As predicted, ^{15}N residence time was higher when birds ate the diet with the lower protein content. Dietary protein content also had a significant effect on $\Delta^{15}N$ ($F_{2,10}=7.53$ and $F_{2,10}=9.58$, $P<0.01$, for cells and plasma, respectively; Fig. 4). $\Delta^{15}N$ did not differ between the diets with low and medium protein, but was significantly lower in the diet with the highest protein content (Fig. 4). Blood, cells and excreta were enriched in ^{15}N relative to diet (one-sample $t>13$, $P<0.01$; Fig. 5). In contrast, urine was either depleted of ^{15}N relative to diet (low protein diet, $t=2.8$, $P<0.05$; Fig. 5) or did not differ significantly from diet (medium and high protein diets, $t=0.4$ and 0.04, $P>0.5$, respectively; Fig. 5).

DISCUSSION

In Yellow-vented bulbuls, the protein content had a significant effect on both the incorporation rate (and hence the residence time) of ^{15}N and the isotopic discrimination factors between tissues and diet. Our results also suggest that for plasma and cells, one-compartment models were better supported than more complex two-compartment models. Here we first discuss the advantages of using model comparison approaches over the graphical reaction progress variable approach proposed by Cerling et al. (Cerling et al., 2007). We then consider the possible physiological mechanisms that lead to both higher ^{15}N incorporation and lower $\Delta^{15}N$ with higher protein intake. Finally, we consider the implications of the effect of protein intake on isotopic incorporation for the interpretation of ecological isotopic data.

One, two, … How many compartments?

Isotopic ecologists have three questions in mind when they conduct an isotopic incorporation experiment. (1) On average, what is the residence time of an isotope in a tissue? (2) How much confidence can we place on this estimate? (3) What are the factors that influence its value? Biologists conduct isotopic incorporation studies on captive animals to answer these three questions. Thus, the central parameter of interest that results from isotopic incorporation experiments is the average retention time (τ), which can easily be transformed into the more widely used half-life $\{t_{1/2}=\tau[\ln(2)]$ (Carleton and Martínez del Rio, 2005)}. In our study, the average retention time of ^{15}N in blood cells was almost fourfold longer than that in plasma. In Yellow-vented bulbuls the isotopic composition of plasma is informative about diet changes at the scale of less than a week, whereas cells reveal patterns of resource use at the scale of from 20 days to a month.

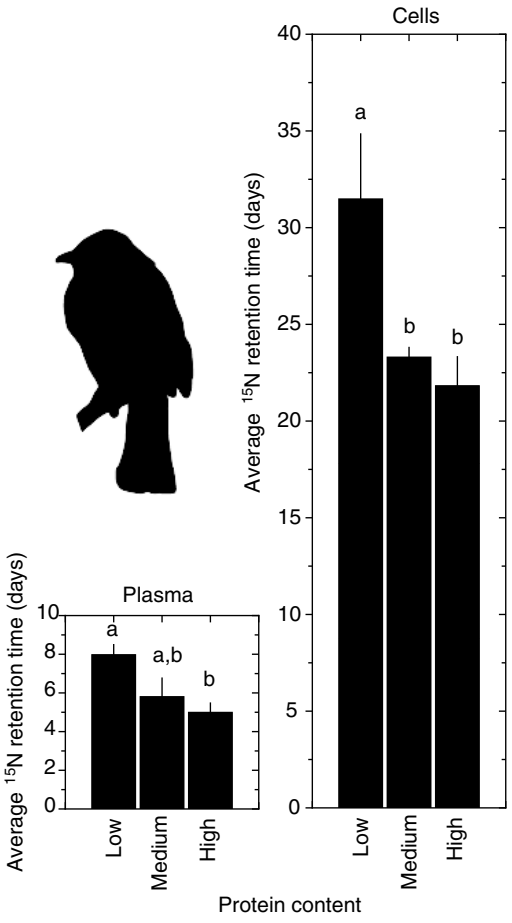

Fig. 3. Dietary protein content had a significant effect on the average residence time of ^{15}N in the plasma solutes and blood cells of Yellow-vented bulbuls. Columns denote means and bars s.e.m. Means with the same letter in each panel are not statistically different from each other. We plotted the data on plasma and cells using the same scale to emphasize the large difference in incorporation rate, and hence residence time, between these two tissues.

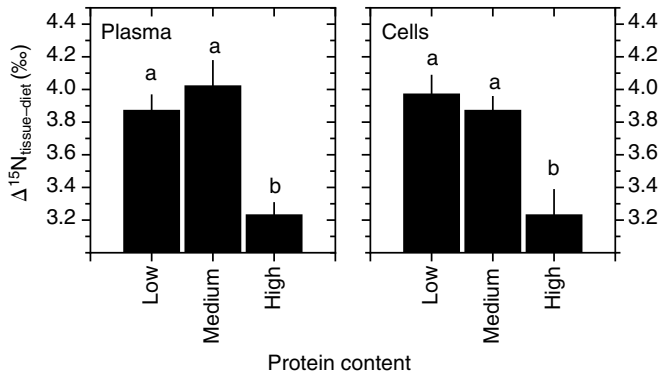

Fig. 4. Dietary protein content had a significant effect on the tissue-to-diet discrimination factor in Yellow-vented bulbuls. Columns denote means and bars s.e.m. Means with the same letter in each panel are not statistically different from each other.

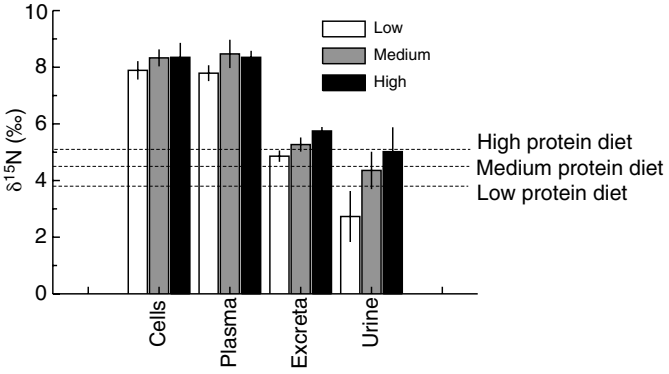

Fig. 5. The δ^{15}N of cells, plasma and excreta was significantly enriched relative to diet in Yellow-vented bulbuls on all diets. In contrast, the δ^{15}N of urine was depleted relative to diet in birds fed the diet with the low protein content. The δ^{15}N of urine did not differ from that of diet in birds fed the diets with the medium and high protein content. Error bars represent 95% confidence intervals for means.

Until recently, most isotopic incorporation studies used first-order, one-compartment models (Eqn 1) to describe isotopic incorporation data (Martínez del Rio and Wolf, 2005). Recently, Cerling et al. (Cerling et al., 2007) questioned the general use of these simple models and proposed the use of an alternative graphical approach to diagnose whether a data set revealed whether models with more than one compartment/pool are needed to describe an isotopic incorporation data set. This method is potentially important because using the wrong model can lead to erroneous estimation of average residence time. Cerling's approach relies on 'linearizing' the isotopic incorporation data and using least-squares linear regression on the resulting linear segments to estimate the relative size of each pool/compartment and its 'decay'/incorporation constant (Fig. 1) (Ayliffe et al., 2004).

Although the need to use the correct model to describe isotopic incorporation data is undeniable, the method proposed by Cerling et al. (Cerling et al., 2007) has several shortcomings: (1) it does not allow an estimate of an isotope's retention time (and a measure of how much confidence we can place in it) to be derived; (2) one has to identify the linear segment for each component/pool visually; (3) it relies on log-transforming data, which often leads to biased estimation (Motulsky and Ransnas, 1987); and (4) there is no quantitative criterion that permits the finding out of whether one should use one, two or more compartments. Using non-linear regression procedures to fit incorporation data to models of increasing complexity overcomes problems 2 and 3 (Bates and Watts, 1988). These models are widely available in most statistical analysis packages. For one-compartment models, the output of these programs includes various estimates of standard error for τ that can then be used to estimate a confidence interval (e.g. Motulsky and Christopoulos, 2003). For more complex, multi-compartment models, Eqn 3 can be used to estimate average retention time (C.M.d.R. and R. A. Sprecher, unpublished observations).

To overcome problem 4 we used the information theoretic approach advocated by Burnham and Anderson (Burnham and Anderson, 2002) and widely adopted in ecological studies (Hobbs and Hilborn, 2006). This approach has a strong theoretical foundation and is based on the idea that we should adopt parsimonious models, which avoid under- and over-fitting and give accurate approximations to the interpretable information in the data available (Anderson and Burnham, 2001). Our data supported the

use of one-compartment over two-compartment models for plasma and blood cells in Yellow-vented bulbuls.

How does protein intake influence isotopic incorporation rate?

In Yellow-vented bulbuls, protein intake had a significant effect on both ^{15}N incorporation rate and $\Delta^{15}N_{tissue-diet}$. Birds that consumed more protein had significantly higher ^{15}N incorporation rates and lower $\Delta^{15}N_{tissue-diet}$. Carleton and Martínez del Rio (Carleton and Martínez del Rio, 2005) hypothesized that protein turnover was a primary determinant of isotopic incorporation. If this hypothesis is correct, then the same factors that influence protein turnover should influence isotopic incorporation. Protein intake influences protein turnover through the action of catabolic (glucagon, adrenaline and cortisol) and anabolic hormones [insulin, IGF and growth hormone (reviewed by Waterlow, 2006)]. The secretion of these hormones appears to be mediated by circulating amino acid concentrations, which in turn are influenced by diet composition (Waterlow, 2006). The differences in ^{15}N incorporation rate among diets observed in Yellow-vented bulbuls is consistent with the idea that protein turnover is a determinant of isotopic incorporation rates.

Muramatsu et al. (Muramatsu et al., 1987) reported an increase in protein turnover with protein intake at modest levels of protein intake in chickens. In these animals, the effect of protein intake on both synthesis and catabolism was independent of protein intake at high protein intakes (Muramatsu et al., 1987). Similarly, in blood cells isotopic incorporation rate (as estimated by ^{15}N retention time) increased from the low to the medium diet, but did not differ between the diets with medium and high protein levels. Tsahar et al. (Tsahar et al., 2005) estimated the maintenance nitrogen requirement (MNR) for Yellow-vented bulbuls as ~8.2 mg N per day. From daily consumption measurements, we estimated that the daily nitrogen intake of birds on the low protein diet was ~97 mg N per day, which is more than an order of magnitude higher than their MNR. We expect the effect of protein intake on isotopic incorporation rate to be greater at lower nitrogen intakes, when N intake rates approach MNR.

Our results were contrary to an assumption widely invoked in the stable isotope literature: if animals satisfy isotopic mass balance, then $\Delta^{15}N_{tissue-diet}$ can only be positive if (1) the $\delta^{15}N$ of excreted nitrogen is more negative than that of tissues (Minagawa and Wada, 1984; Ponsard and Averuch, 1999), and (2) at steady state, the $\delta^{15}N$ of excreted products is equal to that of diet (Martínez del Rio and Wolf, 2005). Although we found that the $\delta^{15}N$ of excreted nitrogen was more negative than that of tissues, in all cases the $\delta^{15}N$ of excreted nitrogen was significantly more positive than that of diet (Fig. 5). The $\delta^{15}N$ of ureteral urine was, as expected, either more depleted of ^{15}N than diet or had the same $\delta^{15}N$ as diet. How can we explain the widely observed positive value of $\Delta^{15}N_{tissue-diet}$ if excreted nitrogen has a more positive value than diet? And, how can we explain the difference in $\delta^{15}N$ between excreta and ureteral urine? There are two alternative/complementary explanations: (1) isotopically light ammonia may have been lost during the collection of excreta samples but not during the collection of ureteral urine, and (2) birds lost isotopically light nitrogen from ureteral urine through an unidentified venue.

Because excreta and urine samples were collected in an HCl solution (pH ~3) which 'traps' ammonia by turning it into ammonium chloride, the first explanation seems unlikely. The second explanation invokes an unknown 'sink' of isotopically light nitrogen. We speculate that this sink is ammonia lost as a gas through respiratory epithelia. Tsahar et al. (Tsahar et al., 2005) demonstrated that in Yellow-vented bulbuls the amount of uric acid and ammonia excreted in ureteral urine is much lower than the amount lost in excreta. These authors suggested that these compounds are re-absorbed in the lower gut as a mechanism of nitrogen conservation. It may be that Yellow-vented bulbuls reabsorb isotopically light uric acid and ammonia preferentially, which would explain the difference between the $\delta^{15}N$ of urine and excreta. Some of the absorbed, isotopically light, ammonia may be then lost in breath. In humans, a significant amount of ammonia is lost in exhaled air, and ammonia levels in breath are routinely measured to diagnose renal diseases and *Helicobacter pylori* infection (Smith et al., 1999; Narasimhan et al., 2001; Kearny et al., 2002). Although this hypothesis is admittedly speculative, it has the virtue of being testable. It requires measuring the contribution of exhaled nitrogen losses to nitrogen balance and the isotopic composition of ammonia in breath. Although the ^{15}N trophic enrichment between tissues and diet is of enormous value to ecologists (Roth and Hobson, 1999; Post, 2002), explaining its magnitude remains an unsolved problem for physiologists (Gannes et al., 1998; Adams and Sterner, 2000; Robbins et al., 2005).

Ecological implications

The rate at which a tissue incorporates the isotopic signal of a diet determines the time window during which ecologists can discern diet changes (Pearson et al., 2003; Podlesak et al., 2005). The almost fourfold difference in isotopic incorporation between plasma and blood cells is useful as it allows the finding out of diets at two contrasting scales. Plasma will reveal the isotopic composition of foods eaten over the last few days, whereas blood cells will reflect the average composition of foods incorporated over approximately a month (Hobson and Clark, 1992; Norris et al., 2004; Dalerum and Angerbjörn, 2005). Blood cells and plasma are particularly valuable tissues in isotopic studies because sampling them is minimally invasive (Norris et al., 2005).

Previous research documented the effect of tissue type (Hobson and Clark, 1992; Dalerum and Angerbjörn, 2005; Podlesack et al., 2005), growth rate (Fry and Arnold, 1982; MacAvoy et al., 2005) and body mass (Carleton and Martínez del Rio, 2005) on isotopic incorporation rate. Our results suggest that the level of dietary protein also plays a role. Although we only documented an effect on plasma and blood cells, two tissues commonly used in ecological studies [Norris et al. (Norris et al., 2005) and references therein], it is likely that protein intake influences the rate of isotopic incorporation in other tissues as well. The effect of protein intake seems to be biologically significant. The average retention time of ^{15}N in birds fed on the low protein diet was longer than that of birds fed on the high protein diet by 136% and 160% for cells and plasma, respectively. Our results support Mirón M. and colleagues' (Mirón et al., 2006) conjecture that protein intake influences isotopic incorporation rates and suggests that the anomalously long isotopic retention times found by Voigt et al. (Voigt et al., 2003) in nectar-feeding bats were the result of an experimental diet with almost no protein. Our results demonstrate the effect of dietary protein on isotopic incorporation in a single species. We hypothesize that this effect may also be found among other species, and that species with low protein intakes such as nectarivores and frugivores will have lower rates of isotopic incorporation than species with high protein intakes, such as carnivores (Tsahar et al., 2006). If our speculation is correct, isotopic field studies may have to be informed by the dietary natural history of the animals studied, including their seasonal diet changes.

LIST OF ABBREVIATIONS AND SYMBOLS

AICc	Akaike's information criteria corrected for small samples
BMR	basal metabolic rate (W)
C	carbon
F	reaction process variable
k	fractional rate of isotopic incorporation (day^{-1})
M_b	mean body mass (g)
MNR	minimal nitrogen requirement (mg N day^{-1})
N	nitrogen
R	ratio of molar abundance of heavy to light isotope
$\delta^{15}N$	$(R_{sample}/R_{standard}-1)\times1000$, where R_{sample} and $R_{standard}$ are $^{15}N/^{14}N$ ratios of the sample and the reference, respectively (‰)
$\Delta^{15}N_{tissue-diet}$	tissue-to-diet discrimination factor (‰)
Δi	difference in AICc ($\Delta i=AICc_i-AICc_{min}$)
τ	estimate of average retention time (days)

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