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Maurine W. Dietz a, Theunis Piersma a b, Anne Dekinga b, Harry Korthals c & Marcel Klaassen c d

a Animal Ecology Group, Centre for Ecological and Evolutionary Studies, University of Groningen, Groningen, The Netherlands
b Department of Marine Ecology, Royal Netherlands Institute for Sea Research (NIOZ), Texel, The Netherlands
c Department of Animal Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands
d Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Geelong, Australia

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Unusual patterns in $^{15}$N blood values after a diet switch in red knot shorebirds

Maurine W. Dietza*, Theunis Piersmaa,b, Anne Dekinga, Harry Korthalsc and Marcel Klaassenc,d

aAnimal Ecology Group, Centre for Ecological and Evolutionary Studies, University of Groningen, Groningen, The Netherlands; bDepartment of Marine Ecology, Royal Netherlands Institute for Sea Research (NIOZ), Texel, The Netherlands; cDepartment of Animal Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands; dCentre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Geelong, Australia

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When a diet switch results in a change in dietary isotopic values, isotope ratios of the consumer’s tissues will change until a new equilibrium is reached. This change is generally best described by an exponential decay curve. Indeed, after a diet switch in captive red knot shorebirds (Calidris canutus islandica), the depletion of $^{13}$C in both blood cells and plasma followed an exponential decay curve. Surprisingly, the diet switch with a dietary $^{15}$N/$^{14}$N ratio ($\delta^{15}$N) change from 11.4 to 8.8 ‰ had little effect on $\delta^{15}$Ni in the same tissues. The diet-plasma and diet-cellular discrimination factors of $^{15}$N with the initial diet were very low (0.5 and 0.2 ‰, respectively). $\delta^{15}$N in blood cells and plasma decreased linearly with increasing body mass, explaining about 40 % of the variation in $\delta^{15}$N. $\delta^{15}$N in plasma also decreased with increasing body-mass change ($r^2 = .07$). This suggests that the unusual variation in $\delta^{15}$N with time after the diet switch was due to interferences with simultaneous changes in body-protein turnover.

Keywords: blood cells; diet switch; discrimination factor; nitrogen-15; plasma; regression model; shorebirds; turnover rate

Introduction

After a diet switch, tissue isotopic ratios will change until they are in equilibrium with the new diet [1–4]. The rate of change in isotopic values is determined by metabolic and growth processes, body size and protein turnover [5] and varies between tissues, with, e.g. low rates in bone and brain, high rates in blood plasma and liver, and intermediate rates in muscles and red blood cells [1,2,6–8]. After a diet switch, the pattern of change in isotopic value in a tissue can generally be described with an exponential decay curve:

$$\delta(t) = \delta(\infty) + (\delta(0) - \delta(\infty)) \cdot e^{-\lambda t},$$

where $\delta(t)$ is the isotopic value of the tissue at time t (in days) after the diet switch, $\delta(\infty)$ is the isotopic value of the tissue in equilibrium with the new diet, $\delta(0)$ is the isotopic value of the tissue in equilibrium with the old diet, and $\lambda$ is the turnover rate of the isotope in the tissue [9]. Only rarely

*Corresponding author. Email: m.w.dietz@rug.nl

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can the change in isotopic value following a diet switch not be described with an exponential decay curve. This may occur when the rate of change is very slow or the period of measurement too short [10]. Although one assumes a single turnover pool when using the exponential decay curve [11,12], there may in fact be more turnover pools even when an exponential decay curve describes the data well [11].

Previously we followed changes in $\delta^{13}C$ in blood cells and plasma of captive red knots (Calidris canutus islandica) after a diet switch [13]. The depletion of $\delta^{13}C$ with time effectively followed exponential decay curves. The decay curves of the isotope indicating protein turnover, $\delta^{15}N$, showed very different patterns. For example, although the new diet had lower $\delta^{15}N$ values, $\delta^{15}N$ of both plasma and blood cells did not decrease to levels below the values of the initial diet until almost 4 months after the diet switch. We here discuss possible causes for these unusual patterns, which may relate to changes in diet-plasma and diet-cellular $^{15}N$ discrimination values ($^{15}N$) that are associated with the depletion and build-up of body stores.

Materials and methods

On 15 January 2002 (= day 0, start of the experiment), seven adult red knots (islandica subspecies, [14]) were caught with mist nets at the island Griend in the Dutch Wadden Sea and brought to the Royal Netherlands Institute for Sea Research (NIOZ, Texel, The Netherlands), where they were kept in outdoor aviaries (l × w × h: 3 × 2 × 2 m) under natural light and temperature conditions (for details of housing conditions, see [13]). Most adult C. c. islandica knots arrive at the wintering grounds in the Dutch Wadden Sea in August (range mid July–September, [15]), where they feed primarily on shellfish [16–18]. Hence, prior to capture, the red knots had likely been on a shellfish diet for about 5 months. We therefore assume that upon capture their tissues were in equilibrium with the shellfish diet. From the start of captivity, red knots were fed ad libitum trout pellets only (Trouvit Classic 2P, Skretting, Hendrix SpA, Italy; composition: crude protein 45 %, carbohydrate 21 %, crude fat 16 %, crude ash 9 %, lysin 3 % indigestible fibres 2 %, phosphorus 1 %), with a different isotopic value than shellfish (see results). Fresh water was available ad libitum.

Upon capture, body mass (± 1 g) was determined and a small blood sample (60–120 μl) was taken by puncturing the wing vein and collected into heparinised capillaries. The capillaries were centrifuged (12 min at 6900g) as soon as possible after sampling, and blood cells and plasma samples were stored in a freezer (−20°C) until transport to the Netherlands Institute of Ecology (NIOO-KNAW), Centre for Limnology, for analysis. In captivity, blood samples were taken every 3 days until 24 January, then once after 5 days and once after 7 days, and from 5 February onwards every 14 days until the last sampling on 8 May 2002. After each sampling the birds were weighed, except on day 3. From the blood samples not only $\delta^{15}N$ of blood cells and plasma were determined, but also $\delta^{13}C$. In contrast to $\delta^{15}N$, $\delta^{13}C$ did change via an exponential decay curve after the diet change [13].

Samples of two favourite shellfish prey species of red knots were also collected in January 2002: cockle (Cerastoderma edule, 9 individuals) and Baltic tellin (Macoma balthica, 15 individuals). In addition, samples of alternative prey species were collected: the small gastropod mudsnail (Hydrobia ulvae, two individuals), and two soft prey species (juvenile Shore Crab, Carcinus maenas, two individuals, and Common Shrimp, Crangon crangon, two individuals). Prey came from the sample locations that were part of the routine annual sampling grid around the island Griend and high tide roosting area Richel in the Dutch Wadden Sea [19]. The sampling grid area forms the foraging area for knots roosting at Griend and Richel [19]. These samples and a sample of the Trouvit diet in captivity were kept in a freezer (−20°C) until transport for analysis to the NIOO-KNAW Centre for Limnology.
Prior to stable isotope analysis, blood cells and plasma samples were freeze dried, and prey and food samples were oven-dried at 50 °C to constant mass. After drying, prey and food samples were powdered in a mortar. Nitrogen stable isotope ratios (parts per thousand, ‰, difference from the 15N/14N ratio in atmospheric N2; further referred to as δ15N) were determined in a EA3000 Eurovector elemental analyser coupled online to an IRMS Delta V Advantage isotope-ratio mass spectrometer via a Confluo III Thermo Scientific interface. Average reproducibility based on replicate measurements of a casein standard (−5.87 ‰) was < 0.2 ‰ (SD).

Statistics were conducted using SPSS for Windows 16.0. Means are presented with SE unless stated otherwise. To allow for a robust analysis of the impacts of body mass (change) and (changes in) body composition on δ15N we reanalysed published data on the complex relationship between total fat content and body mass in red knots [20–22]. Previously, it was shown that in red knots the relationship between total fat mass and body-mass changes abruptly at a certain body mass; total fat mass increased strongly from a body mass of about 120 g onward in Calidris canutus canutus [20,21] and from 133 g onward in Calidris canutus rufa [22]. It can be expected that body-protein turnover will also change around this body mass. Because the point of change arrived at different body-mass values in the two subspecies, we combined both data sets and fitted a simple and extended continuous piecewise regression model through the data using the nonlinear regression algorithm procedures from the NONLIN 2.5 package (shareware program, P.H. Sherrod, based on the nonlinear least-squares algorithm described in [23]). The following simple piecewise regression model was used:

\[
Y = a + b_1 X - r(b_1 - b_2) \ln(1 + e^{((X - c)/r)})
\]

where a is the intercept, \(b_1\) is the slope of the first part, \(b_2\) is the slope of the second part of the regression, c is the estimated breakpoint between the two phases, and r is a smoothness parameter set at 0.5 [24]. The extended model, used to describe the data with two piecewise regressions (one for each group) in one model, was

\[
Y = d_1 + b_1 X - r(b_1 - b_2) \ln(1 + e^{((X - c)/r)})
+ d_2 + b_1 X - r(b_1 - b_2) \ln(1 + e^{((X - c)/r)})
\]

Figure 1. The relationship between body mass and total amount of body fat for individuals of the red knot subspecies C. c. rufa (closed circles) and C. c. canutus (open circles). The piecewise regression line had an intercept at −9.877 ± 7.193 g and a point of change at 129 ± 3 g. At that mass, the increase in total fat mass with body mass increased from 0.151 ± 0.066 g g\(^{-1}\) to 0.803 ± 0.035 g g\(^{-1}\).
where \( d_1 \) and \( d_2 \) were dummy variables for the two groups. The piecewise regressions did not differ between subspecies (the simple model described the data best; \( F_{4,89} = 1.41, p > 0.05 \)), and the common point of change was at a body mass of 129 ± 3 g (Figure 1). At that mass, the increase in total fat mass with body mass changed from 0.15 ± 0.07 g g\(^{-1}\) to 0.80 ± 0.04 g g\(^{-1}\), a more than fivefold increase. Therefore, the analyses of the impacts of body mass (change) and (changes in) body composition on \( \delta^{15}N \) were done for birds with body masses ≤ 129 and > 129 g separately.

**Results**

\( \delta^{15}N \) did not differ between the shellfish prey species (cockles 11.5 ± 0.2‰ and Baltic tellins 11.4 ± 0.3‰; ANOVA \( F_{1,22} = 0.05, p = .83 \)). Mean shellfish \( \delta^{15}N \) (11.4 ± 0.2‰) was higher than in Trouvit (8.8 ± 0.2‰; ANOVA \( F_{1,24} = 18.71, p < .001 \)). The \( \delta^{15}N \) of the alternative hard-shelled prey, the mudsnail, did not differ from shellfish (11.2 ± 0.1‰; ANOVA \( F_{2,23} = 0.10, p = .90 \)), while the alternative soft prey, juvenile Shore Crab and Common Shrimp, had a ca. 2‰ higher \( \delta^{15}N \) than the hard-shelled prey species (13.7 ± 0.8‰; Anova \( F_{3,28} = 12.98, p < .001 \), post hoc Tukey test).

Body masses of the red knots decreased during the first days in captivity, from 159 ± 3 g (n = 7) upon capture to 126 ± 7 g (n = 5) at day 6 (Figure 2(a)). Thereafter body mass increased rapidly.
Figure 3. Variation in mean ($\pm$ SE) $\delta^{15}$N in blood cells ($\delta^{15}$N$_{\text{cells}}$, closed circles, solid lines) and plasma ($\delta^{15}$N$_{\text{plasma}}$, open circles, dashed lines) in red knots (C. c. islandica) with body mass ($m_b$). Data were analysed for birds with body masses $\leq 129$ and $> 129$ g separately. When body mass was $\leq 129$ g, no significant linear relationships were found. For body masses $> 129$, slopes were equal but intercepts differed between individuals. The general linear regression model for blood cells is $\delta^{15}$N$_{\text{cells}} = 14.59 (\pm 0.67) - 0.02 (\pm 0.004) m_b$, $n = 62$, $r^2 = .41$, $p < .001$ (individual intercept parameters: $-0.13$, $-0.11$, $0.60$, $-0.40$, $0.47$, $0.37$, $0.00$); and for plasma $\delta^{15}$N$_{\text{plasma}} = 17.18 (\pm 1.12) - 0.04 (\pm 0.01) m_b$, $n = 61$, $r^2 = .39$, $p < .001$ (individual intercept parameters: $0.52$, $0.33$, $1.22$, $-0.85$, $0.59$, $0.25$, $0.00$).

to stabilise at around $143 \pm 3$ g ($n = 35$, day range 14–63). Towards the end of the experiment, the spring fuelling period started and body mass increased again.

Blood cell and plasma $\delta^{15}$N varied with time, but an exponential decay curve clearly did not describe the decline in $\delta^{15}$N (Figure 2(b)). Confirming that prior to capture the birds were in equilibrium with the shellfish diet, at capture, $\delta^{15}$N values were well below the alternative soft prey levels and slightly above shellfish levels. The diet-plasma and diet-cellular $^{15}$N values on the initial shellfish diet were very low (0.2 and 0.5 ‰, respectively). Initially, $\delta^{15}$N of blood cells and plasma did not differ (paired Student's t-test, $t_5 = -0.65$, $p = .54$), but thereafter plasma had higher $\delta^{15}$N values than blood cells. After the diet switch, $\delta^{15}$N showed an increase followed by a decrease, whereupon it stabilised for about 50 days while remaining above the shellfish value (mean $\delta^{15}$N for day range 14–63 was $11.8 \pm 0.1$ and $12.3 \pm 0.1$‰ for cells and plasma, respectively). Thereafter, another increase–decrease bout occurred. The increments were more pronounced in plasma than in blood cells. Only at the end of the experiment, when the birds were getting heavy, $\delta^{15}$N finally depleted to values below the shellfish value. At that time, 113 days after the diet switch, blood cells and plasma were 1.9 and 1.7 ‰ higher than the Trouvit diet, respectively.

Surprisingly, the diet switch had little effect on $\delta^{15}$N levels. The increments in $\delta^{15}$N coincided with body mass decreases or start of body-mass increase, suggesting that simultaneous changes in body-protein turnover may have interfered with $\delta^{15}$N. In both blood cells and plasma, $\delta^{15}$N decreased linearly with increasing body mass (Figure 3). However, this relationship was not significant at body masses $\leq 129$ g, where body composition is characterised by a lower fat content (Figure 1). This lack of relationship may, however, also be due to the relatively small body-mass range. When body mass exceeded 129 g, and body fat was higher, significant linear relationships were found for which the intercepts differed between individuals, but slopes did not (univariate analysis of variance, blood cells: $F_{6,54} = 3.83$, $p < .01$ and $F_{6,48} = 0.22$, $p = .97$, respectively; plasma: $F_{6,53} = 2.72$, $p < .05$ and $F_{6,47} = 2.28$, $p = .052$, respectively). The decrease in $\delta^{15}$N with body mass was twice as steep in plasma as in blood cells ($-0.04 \pm 0.01$‰ g$^{-1}$ and $-0.02 \pm 0.004$‰ g$^{-1}$, respectively). The $r^2$ of the final models were .41 and .38 for blood cells and plasma, respectively, indicating that body mass explained about 40% of the variation in $\delta^{15}$N.
Figure 4. The relationship between mean (± SE)$\delta^{15}$N in blood cells ($\delta^{15}$N$_{\text{cells}}$, closed circles) and plasma ($\delta^{15}$N$_{\text{plasma}}$, open circles) in red knots (C. c. islandica) with daily body-mass change ($\Delta m$) at body masses (a) ≤ 129 g and (b) > 129 g. For body mass ≤ 129 g, no significant linear relationships were found. For body mass > 129, there was a significant linear relationship for plasma. Slopes and intercepts did not differ between individuals (p = .91 and .44, respectively). The linear relationship was $\delta^{15}$N$_{\text{plasma}} = 12.34 (± 1.13) - 0.15 (± 0.08) \Delta m$, n = 55, $r^2 = .07$, p = .04.

$\delta^{15}$N showed less correlation with daily body-mass change. Again, no significant relationships were found when body mass was ≤ 129 g (Figure 4(a)). At body masses > 129 g, slopes and intercepts did not differ between individuals (for blood cells: $F_{5,42} = 1.54, p = 0.20$ and $F_{6,47} = 1.90, p = 0.10$, respectively; for plasma: $F_{5,42} = 0.31, p = 0.91$ and $F_{6,47} = 0.38, p = 0.38$, respectively). However, only for plasma a significant linear relationship was found (p < 0.05, for cells p = 0.32; Figure 4(b)). Body-mass change explained only little of the variation in $\delta^{15}$N in blood plasma ($r^2 = .07$).

Discussion

There are two remarkable findings in the pattern of change over time in $\delta^{15}$N in blood cells and plasma after a diet switch in red knots: (1) the very low diet-plasma and diet-cellular $^{15}$N values with the old diet and (2) the lack of the expected depletion pattern with time. In their review, Caut et al. [25] presented mean diet-whole blood and diet-plasma $^{15}$N values for birds of 2.25 ± 0.20 and 2.82 ± 0.14‰, respectively. Obviously, with values of 0.2 and 0.5‰ the diet-cellular and diet-plasma $^{15}$N values in red knots were very low.

Martinez del Rio et al. [5] summarised four, sometimes contradicting, predictions for the physiological factors that may influence $^{15}$N values: the discrimination factor should (1) decrease with increased protein quality of the diet (protein quality hypothesis); (2) increase with protein
content of the diet (protein quantity hypothesis); (3) decrease with the efficiency of nitrogen deposition measured as the ratio between protein assimilation and protein loss; and (4) increase with fasting time (notably during starvation, [26]). Especially the protein quality and quantity hypotheses are considered to be critical [27]. Therefore, because the birds were not growing and we also lack information on nitrogen deposition efficiency in red knots (prediction 3), and because the birds were not starved (prediction 4), we hereafter focus on the protein quality and protein quantity hypotheses.

Prediction (1) is based on an analysis by Robbins et al. [28] showing that $^{15}$N decreases with trophic level from herbivores to carnivores, thus with increasing (biological) protein quality. Red knots are carnivores and the low $^{15}$N is hence possibly related to their diet. If this is the case, the very low diet-plasma and diet-cellular $^{15}$N values imply that the dietary protein source (shellfish) perfectly matched their metabolic demand for indispensable amino acids [29]. This, however, seems unlikely. Also, diet-plasma and diet-whole blood $^{15}$N values in the related and likewise carnivorous Dunlin (Calidris alpina pacifica) were within the expected range (3.3 and 3.0 ‰ respectively, on an artificial marine or terrestrial diet [30]). Moreover, a recent survey found no effect of foraging guild (eight guilds of birds, among which herbivores and carnivores) on $^{15}$N and $^{13}$C within a tissue (10 tissues, among which blood and plasma [31]).

In contrast to prediction (1), prediction (2) implies that $^{15}$N will increase with trophic level [26, 28]. Prediction (2) is mainly based on Pearson et al. [32] who gave yellow-rumped warblers (Dendroica coronata) diets that differed in N-content (varying from 2.9 to 8.4 ‰), Focken [33] who fed Nile tilapia (Oreochromis nilotus) different quantities of a diet containing 40.2 ‰ crude protein (5, 10 and 20 g kg$^{-0.8} d^{-1}$), and the interspecific study of McCutchan et al. [26]. The shellfish diet of the red knots contained a high amount of protein (ca. 75 ‰ protein of ash-free dry mass; data for Baltic tellin collected in January in the Dutch Wadden Sea [34,35]). Following prediction (2), diet-plasma and diet-cellular $^{15}$N could thus only be very low if the amount of food eaten would have been (very) low [33]. This is highly unlikely considering that the knots were captured in winter (January) when they have the highest thermoregulation costs [36] and thus are expected to have a high food intake. Hence, this study does not support the protein quantity hypothesis.

All studies and reviews referred to above consider only data collected in captive animals. Here, we considered diet-plasma and diet-cellular $^{15}$N of wintering wild knots entrained on much higher energy expenditure rates than when kept in captivity. Metabolic rate may affect stable isotope turnover rates and discrimination factors because metabolic processes contribute to tissue turnover, but the effects found are unclear [37]. In an interspecific comparison, MacAvoy et al. [38] found a positive correlation between the turnover rates of $\delta^{15}$N and $\delta^{13}$C and metabolic rate. But in intraspecific comparisons, nitrogen and carbon turnover rates and discrimination factors were not affected by differences in metabolic rate [9,37,39]. These intraspecific findings suggest that the low diet-plasma and diet-cellular $^{15}$N found were not due to the high-energy expenditure of the red knots in the wild.

So far, reported turnover patterns of $^{13}$C and $^{15}$N were rather similar within tissues (e.g. [30,38, 40]), although turnover rates may differ (e.g. [9,41]). In red knots, the turnover pattern of blood cell and plasma $\delta^{15}$N was completely dissimilar from that of $\delta^{13}$C in the same tissue samples [13] and neither followed an exponential decay curve nor a multi-compartment model. The initial increase and decrease of $\delta^{15}$N coincided with the initial decrease and increase in body mass, the final decrease in $\delta^{15}$N coinciding with the onset of the fuelling period. This indicates that a change in body-protein turnover may have affected the turnover pattern of $\delta^{15}$N, which is confirmed by the negative relationships between $\delta^{15}$N in blood cells and plasma and body mass, and between $\delta^{15}$N in plasma and body-mass change.

Turnover rates of $\delta^{15}$N have been shown to decrease with decreasing protein content of the food in nectarivorous bats [42] and frugivorous birds [43]. Although the diets of the knots in the
wild and captivity differed in protein content, both diets contained high amounts of protein (45 % crude protein in Trouvit and 75 % protein of ash-free dry mass in Baltic tellin [34,35]). Many red knots have been kept at our facility on the trout diet for over 15 years without health or moulting problems. It seems therefore unlikely that the turnover patterns of $\delta^{15}$N were constraint or biased by the protein content of the diets. Moreover, turnover rate seems to have increased instead of decreased, because $\delta^{15}$N stabilised already 13 days after the diet switch (after the initial increase and decrease in $\delta^{15}$N immediately after the diet switch; Figure 1), which is much faster than the turnover of $\delta^{13}$C in the same samples, which had half-lives of 6.03 and 15.07 days in plasma and blood cells, respectively [13]. Note that because we do not know if the tissues were in equilibrium with the Trouvit diet, the differences found in the stable period cannot be considered representing discrimination factors.

Various studies using stable isotope analysis have shown $\delta^{15}$N enrichment of tissues in nutritionally stressed animals (e.g. [3,44,45]; but see e.g. [46,47]). Judging from the relationships between $\delta^{15}$N values and body mass and body-mass change, this also appears to be the case in our study, at least partly explaining the aberrant turnover pattern observed in $\delta^{15}$N in blood cells and plasma after the diet switch. That only little of the variation was explained in a direct comparison between $\delta^{15}$N in blood cells and plasma with body-mass change may be explained by the complexity of the interacting turnover and enrichment processes that were differentially affected by the body-mass decrease following a diet switch.

Our findings raise several questions. To help solving these, the experiment could be repeated while including protein quality and quantity measurements and extending it with a controlled version in which captive birds live on a shellfish diet prior to the diet switch. In this controlled situation, body-mass variations at various points in time can be induced via food restriction. This could shed more light on the possible differences between free-living and captive knots in diet-plasma and diet-cellular $^{15}$N and turnover patterns of $\delta^{15}$N, and on the effect of body-mass changes on diet-plasma and diet-cellular $^{15}$N and turnover patterns of $\delta^{15}$N. Measuring protein quality and quantity adds to a further understanding of the biological basis for the patterns. Protein quantity is easily measured, but determining protein quality is difficult. Protein quality is estimated as the concentration of the essential amino acids as percentage of the diet’s crude protein [27]. The amino-acid requirements of wild animals are largely unknown, forcing, e.g. Florin et al. [27] in a recent overview on birds and mammals to fall back on data of laboratory rats. This problem should be solved first to make further research more valuable.

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