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Single-point isotope measurements in blood cells and plasma to estimate the time since diet switches

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Summary

1. Understanding ecological phenomena often requires an accurate assessment of the timing of events. To estimate the time since a diet shift in animals without knowledge on the isotope ratios of either the old or the new diet, isotope ratio measurements in two different tissues (e.g. blood plasma and blood cells) at a single point in time can be used. For this 'isotopic-clock' principle, we present here a mathematical model that yields an analytical and easily calculated outcome.

2. Compared with a previously published model, our model assumes the isotopic difference between the old and new diets to be constant if multiple measurements are taken on the same subject at different points in time. Furthermore, to estimate the time since diet switch, no knowledge of the isotopic signature of tissues under the old diet, but only under the new diet is required.

3. The two models are compared using three calibration data sets including a novel one based on a diet shift experiment in a shorebird (red knot *Calidris canutus*); sensitivity analyses were conducted. The two models behaved differently and each may prove rather unsatisfactory depending on the system under investigation. A single-tissue model, requiring knowledge of both the old and new diets, generally behaved quite reliably.

4. As blood (cells) and plasma are particularly useful tissues for isotopic-clock research, we trawled the literature on turnover rates in whole blood, cells and plasma. Unfortunately, turnover rate predictions using allometric relations are too unreliable to be used directly in isotopic-clock calculations.

5. We advocate that before applying the isotopic-clock methodology, the propagation of error in the 'time-since-diet-shift' estimation is carefully assessed for the system under scrutiny using a sensitivity analysis as proposed here.

Key-words: isotopic clock, mathematical model, sensitivity analysis, stable carbon isotope, timing of events

Introduction

Reaching an understanding of ecological phenomena often requires an accurate assessment of the timing of events. For example, the timing of reproduction has a large impact on the future prospects of offspring (Brinkhof *et al*. 1993; Varpe *et al*. 2007); the mismatch between timing of breeding and the food peak is a major contributor to the climate change effect on reproductive success (Both & Visser 2001; Visser, Holleman & Gienapp 2006; Tulp & Schekkerman 2008) and temporal patterns of predator–prey interactions must be known to assess and understand the effects of predation risk (Roth & Lima 2007). However, in many cases the timing of events is difficult to ascertain. When events that require timing involve a change of diet, time estimates may be obtained from the change in stable isotope ratios in tissues.

After a diet switch, body tissues adopt the isotopic ratio of the new diet. The rate at which this happens varies from tissue to tissue. If one knows the isotopic ratio of the original diet and of the new diet, as well as the rate at which a tissue turns

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over, one can calculate from a single measurement how long ago an animal has switched diet (see Guelinckx *et al.* 2008, on movement patterns in a marine fish). The only prerequisite is that the measurement is conducted before the tissue has completely equilibrated to the new diet. Hesslein, Hallard & Ramlal (1993) proposed that when there is incomplete knowledge of either the old or the new diet, using isotope ratio measurements in two different tissues at one point in time can also be used to calculate the time since a diet switch. An additional requirement for this isotopic-clock principle is that the two tissues should be sufficiently different in their rate of turnover. For these methods, carbon stable isotope ratios are highly suitable, but any other isotope or alternative marker could also be used as long as it changes in the tissues in response to a diet shift and the turnover rates in the tissues are known.

Although Hesslein *et al.*'s (1993) idea was proposed more than 15 years ago, only recently Phillips & Eldridge (2006) provided the mathematical equations allowing the estimation of the timing of the diet switch from a single-point isotopic measurement in two different tissues. Their dual-tissue model, however, suffers from three limitations. First, it only allows a numerical solution, which makes it slightly cumbersome to calculate the timing of a switch. Secondly, their numerical solutions allow the difference between the stable isotope ratios of the old and new diets to vary across a data set, which we consider generally unrealistic. Finally, their model requires knowledge on the isotopic signature of the consumers' tissues in equilibrium with the old diet. This signature is not always easily obtainable (e.g. when dealing with migratory birds arriving from a poorly defined origin at a new stopover site).

Here, we present an alternative dual-tissue model that yields a straightforward and easily calculated outcome. Furthermore, it assumes the isotopic difference between the old and new diets to be constant if multiple measurements are taken on the same subject at different points in time. Finally, it requires no knowledge of the isotopic signature of the consumers' tissues in equilibrium with the old diet, but one should know the signature of the tissues in equilibrium with the new diet. We illustrate this theoretical model with an empirical model, that of the long-distance migrant shorebird red knot (*Calidris canutus* subspecies *islandica*) in which we collected blood cells and plasma after a diet switch. Blood cells and plasma differ in turnover rates (Hobson & Clark 1993) and are easily obtained thus yielding a high potential for use in the field.

Using the red knot data and the two calibration sets used by Phillips & Eldridge (2006), we will compare our new model with that proposed by Phillips and Eldridge. We performed a simple sensitivity analysis for the two models to test the robustness of the results, also comparing them with results from a single-tissue model relying on the isotopic ratios of both the original and new diets. In the 'Discussion', we discuss the pros and cons of the models. Furthermore, using data from the literature, we assess the general potential of stable isotope ratios in blood components as a chemical clock across mammals and birds.

Materials and methods

ISOTOPIC-CLOCK MODEL

The change in the isotopic signature of a tissue following a diet switch can be described by an exponential decay curve as:

$$\delta(t) = \delta(\infty) + (\delta(0) - \delta(\infty)) \times e^{-\lambda \cdot t}, \tag{eqn 1}$$

where $\delta(t)$ is the isotopic signature of the tissue at time t after the diet switch (t in days), $\delta(\infty)$ is the isotopic signature of the tissue in equilibrium with the new diet [i.e. $\delta(\infty) = \delta_{new} + \Delta_{new}$, where δ_{new} is the isotopic signature of the new diet and Δ_{new} is the discrimination factor between the tissue and new diet], $\delta(0)$ is the isotopic signature of the tissue in equilibrium with the old diet [i.e. $\delta(0) = \delta_{old} + \Delta_{old}$, where δ_{old} is the isotopic signature of the old diet and Δ_{old} is the discrimination factor between the tissue and old diet] and λ is the turnover rate of the isotope in the tissue as a result of metabolic and growth processes.

Equation 1 can be rearranged to read as:

$$t = \frac{\ln\left(\frac{\delta(0) - \delta(\infty)}{\delta(t) - \delta(\infty)}\right)}{\lambda}. \tag{eqn 2}$$

If one knows both $\delta(0)$ and $\delta(\infty)$, eqn 2 can thus be used to calculate time since diet switch from a single isotopic measurement from a single tissue (e.g. Guelinckx *et al.* 2008; Oppel & Powell 2010). If either $\delta(0)$ or $\delta(\infty)$ is unknown, another approach is required using a single measurement in time in two tissues with different turnover rates. If the isotopic signature is measured in two different tissues, 1 and 2, eqn 1 can be written for each tissue as:

$$\delta(t)_1 = \delta(\infty)_1 + (\delta(0)_1 - \delta(\infty)_1) \times e^{-\lambda_1 \cdot t}, \tag{eqn 3a}$$

$$\delta(t)_2 = \delta(\infty)_2 + (\delta(0)_2 - \delta(\infty)_2) \times e^{-\lambda_2 \cdot t}. \tag{eqn 3b}$$

This set of exponential decay curves can be rearranged to read as:

$$\delta(0)_1 - \delta(\infty)_1 = \frac{\delta(t)_1 - \delta(\infty)_1}{e^{-\lambda_1 \cdot t}}, \tag{eqn 4a}$$

$$\delta(0)_2 - \delta(\infty)_2 = \frac{\delta(t)_2 - \delta(\infty)_2}{e^{-\lambda_2 \cdot t}}. \tag{eqn 4b}$$

Assuming that the differences in isotopic signature of the two tissues in equilibrium with the old and new diets, that is, $\delta(0)_1 - \delta(\infty)_1$ and $\delta(0)_2 - \delta(\infty)_2$, are identical, eqn 4a can be set equal to eqn 4b. Next, this equality can be solved for t, which results in the following isotopic-clock model:

$$t = \frac{\ln\left(\frac{\delta(t)_1 - \delta(\infty)_1}{\delta(t)_2 - \delta(\infty)_2}\right)}{\lambda_2 - \lambda_1}. \tag{eqn 5}$$

Why we arrive at an analytical solution of this problem, whereas Phillips & Eldridge (2006) did not, is because of the fact that we

defined the change in equilibrium isotopic signatures of the tissues as $\delta(0) - \delta(\infty)$, whereas they defined it as $\delta(\infty) - \delta(0)$. Their slightly different approach has a considerable mathematical consequence in that theirs does not result in an analytical solution but in a system of two nonlinear equations that can only be solved numerically.

Our model, just as the model of Phillips & Eldridge (2006), builds on two assumptions. First, it assumes that the incorporation of stable isotopes in the consumers' tissues can be described adequately by first-order, one-compartment models (eqn 1). Secondly, it assumes that the difference in isotopic signature of the two tissues in equilibrium with the two diets does not differ between tissues. In other words, any difference in discrimination factor (Δ) between the two diets in tissue 1 is mirrored in tissue 2. Mathematically, this is presented as:

$$\delta(0)_1 - \delta(\infty)_1 = \delta(0)_2 - \delta(\infty)_2, \qquad \text{(eqn 6a)}$$

which is equivalent to

$$\Delta_{old,1} - \Delta_{new,1} = \Delta_{old,2} - \Delta_{new,2}. \qquad \text{(eqn 6b)}$$

Although this assumption is necessary to derive an analytical solution, it is not always entirely true (Hobson & Clark 1992). Violation of this assumption will result in an under- or overestimate of the time since diet switch, depending on the magnitude of the difference between the various diet-tissue discrimination factors. This becomes more important when the relative value of the discrimination factor is high relative to the absolute difference between δ_{old} and δ_{new}.

ANIMALS AND EXPERIMENTAL PROCEDURE

Seven adult red knots of the *islandica* subspecies were caught with mistnets in the Dutch Wadden Sea on 15 January 2002 (= day 0, start of the experiment) and brought to the Royal Netherlands Institute for Sea Research (NIOZ, Texel, The Netherlands). The knots were kept in outdoor aviaries ($l \times w \times h$: $3 \times 2 \times 2$ m) under natural light and temperature conditions. Each aviary had a small, barren artificial mudflat to allow knots to practice their probing activity. The floor was continuously flushed with fresh sea water to prevent foot diseases and fresh water was available *ad libitum*. The majority of adult *C. c. islandica* knots arrive in August in the Dutch Wadden Sea (range: mid July to September; Nebel *et al.* 2000), where they feed mostly on shellfish (Dekinga & Piersma 1993; van Gils *et al.* 2003). Therefore, we assume that in January the red knots had been on a shellfish diet for *c.* 5 months before capture so that their tissues were in equilibrium with their shellfish diet. From the start of captivity, knots were fed *ad libitum* trout pellets (Trouvit Classic 2P; Skretting, Hendrix SpA, Italy), which have a different isotopic signature than shellfish (see 'Results'). An experienced captive knot was placed with the new birds to encourage them to eat the new diet.

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 heparinized capillaries. After arrival at NIOZ, 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. The birds were weighed after each sampling (± 1 g), except on day 3 as a result of a misunderstanding. The capillaries were centrifuged (12 min at 6900 *g*) as soon as possible after sampling to separate plasma from blood cells. 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.

Individuals of two favourite prey species of red knots were also collected in January 2002: the cockle (*Cerastoderma edule*, 10 individuals) and baltic tellin (*Macoma balthica*, 13 individuals). The samples were kept in a freezer (-20 °C) until transport for analysis together with a sample of the diet in captivity, Trouvit, to the NIOO-KNAW, Centre for Limnology.

STABLE ISOTOPE ANALYSES

Prior to the 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. Carbon stable isotope ratios (parts per thousand, ‰, difference from the $^{13}C/^{12}C$ ratio in Vienna PeeDee limestone; further referred to as $\delta^{13}C$) were determined in a Carlo Erba 1106 elemental analyser coupled online to a Finnigan Delta S isotope-ratio mass spectrometer via a Finnigan con-flo interface. Average reproducibility based on replicate measurements was < 0.2‰.

VALIDATION OF THE MODEL

We studied the performance of our model and that of Phillips & Eldridge (2006) using our knot data and the two data sets that they used. The latter two data sets report on the changes in $\delta^{13}C$ in various tissues of captive-kept adult quail (*Coturnix japonica*) and adult gerbils (*Meriones unguienlatus*) after a diet switch. The quail data set comprises of regular $\delta^{13}C$ measurements after a diet switch of liver, blood and bone (Hobson & Clark 1992) and in the gerbil samples of liver, muscle and hair were taken at regular time intervals after a diet switch (Tieszen *et al.* 1983). Using $\delta^{13}C$ measurements in different pairs of fast and slow turning-over tissues (i.e. liver–blood, liver–bone and blood–bone in quail; liver–muscle, liver–hair and muscle–hair in gerbil; blood plasma–blood cells in knot), we compared the actual time with the estimated time since the diet switch using both our own model (eqn 5) and that of Phillips and Eldridge. For the latter method we did not recalculate the time estimates since diet switch for gerbil and quail, but rather used the original time estimates as presented in Phillips & Eldridge's (2006) original study. Similar to Phillips and Eldridge, we also used the turnover rates of the various tissues (λ) as provided in the respective quail and gerbil studies (see Appendix S1 in Supporting Information).

SENSITIVITY ANALYSIS

As a result of measurement errors and biological variation, all parameters in both our and Phillips and Eldridge's model are inherently associated with error. To exemplify the sensitivity of both models in their calculation of t for this variation in their parameter estimates, we conducted a simple uncertainty analysis using Monte Carlo simulation. We based this analysis on our blood plasma and cells data of knots and on data from Hobson & Clark (1992) on quail using liver (which has a comparable turnover rate to plasma) and blood (which has a comparable turnover rate to blood cells) as the fast and slow turning-over tissues, respectively.

Hobson and Clark established in their experiment the following equations for the turnover of $\delta^{13}C$ in liver:

$$\delta(t)_1 = -20.67 - 2.90e^{-0.272t} \qquad \text{(eqn 7)}$$

and blood:

Table 1. Parameters and their error terms used in the Monte Carlo simulations to study the propagation of variation in parameter estimates in the estimation of the time since diet switch using the model presented in this study (eqn 5) and the model presented by Phillips & Eldridge (2006). Parameter means and error terms are based on a study in quail (Hobson & Clark 1992) and red knot (this study). Turnover rates (λ) and carbon isotope ratios [$\delta(t)$] were measured in liver (quail tissue 1) and blood (quail tissue 2) or plasma (knot tissue 1) and blood cells (knot tissue 2) prior to [$\delta(0)$] and after a diet switch until the tissue again reached the new equilibration carbon isotope ratio [$\delta(\infty)$]

| | Quail | | Red knot | | | Relevant for the model in: | |
	Mean	Error	Mean	Error	SD or SE	This study	Phillips & Eldridge (2006)
Parameter							
λ_1	$0 \cdot 272 \, \text{day}^{-1}$	$0 \cdot 038$	$0 \cdot 144 \, \text{day}^{-1}$	$0 \cdot 028$	SE	Yes	Yes
λ_2	$0 \cdot 062 \, \text{day}^{-1}$	$0 \cdot 006$	$0 \cdot 046 \, \text{day}^{-1}$	$0 \cdot 003$	SE	Yes	Yes
$\delta(t)_1$	Eqn 7	$0 \cdot 4$	Eqn 9	$0 \cdot 283$	SD	Yes	Yes
$\delta(0)_1$	$-23 \cdot 68\text{‰}$	$0 \cdot 144$	$-23 \cdot 5\text{‰}$	$0 \cdot 107$	SE	No	Yes
$\delta(\infty)_1$	$-20 \cdot 67\text{‰}$	$0 \cdot 144$	$-19 \cdot 7\text{‰}$	$0 \cdot 107$	SE	Yes	No
$\delta(t)_2$	Eqn 8	$0 \cdot 4$	Eqn 10	$0 \cdot 111$	SD	Yes	Yes
$\delta(0)_2$	$-23 \cdot 38\text{‰}$	$0 \cdot 144$	$-21 \cdot 6\text{‰}$	$0 \cdot 042$	SE	No	Yes
$\delta(\infty)_2$	$-20 \cdot 41\text{‰}$	$0 \cdot 144$	$-18 \cdot 4\text{‰}$	$0 \cdot 042$	SE	Yes	No

$$\delta(t)_2 = -20.41 - 2.85e^{-0.062t}. \qquad \text{(eqn 8)}$$

These equations and similar equations for turnover in δ^{13}C in red knot blood plasma and cells (see eqns 9 and 10 in 'Results') were used to calculate $\delta(t)_1$ and $\delta(t)_2$ for a range of t values varying between 1 and 200 days (in 21 equidistant steps on a logarithmic scale). Subsequently for each t (further denoted by t_{actual}), a Monte Carlo analysis was conducted where for each parameter in Table 1 a random value was drawn from a normal distribution defined by their mean and error term, after which time since diet switch was calculated using both Phillips and Eldridge's model ($t_{\text{P\&E}}$) and our model ($t_{\text{this study}}$). For each t_{actual} this procedure was repeated 2000 times, after which the distributions of $t_{\text{P\&E}}$ and $t_{\text{this study}}$ were established and compared.

For the various parameters we used either SE or SD as the error term. In a standard research scenario, $\delta(t)_1$ and $\delta(t)_2$ are measured, where for each pair of these measurements an investigator wants to estimate $t_{\text{P\&E}}$ or $t_{\text{this study}}$. The distributions of $\delta(t)_1$ and $\delta(t)_2$ are assumed to be normal and are thus determined by their mean $\pm$ SD. To calculate $t_{\text{P\&E}}$ or $t_{\text{this study}}$ from $\delta(t)_1$ and $\delta(t)_2$ a number of additional parameters are required (listed in Table 1). In the standard research scenario, these parameters are not estimated simultaneously with $\delta(t)_1$ and $\delta(t)_2$ but measured at another occasion or derived from the literature. These parameters are actually means that are not precisely known having a (presumed) normal distribution determined by their mean $\pm$ SE.

The values for the various error terms for red knot and quail are depicted in Table 1. Data for quail were derived from Hobson & Clark's (1992) original study. For λ_1 and λ_2, they did not present the SEs but we approximated these from data presented in their original study. Phillips and Eldridge's model uses information on the equilibration δ^{13}C of the tissues prior to the diet switch [i.e. $\delta(0)_1$ and $\delta(0)_2$], whereas our model uses the equilibration δ^{13}C of the tissues after the diet switch [i.e. $\delta(\infty)_1$ and $\delta(\infty)_2$]. To avoid any bias in the sensitivity analysis of the two models for differences in the estimation precision of these parameters, we used the overall average SE for $\delta(0)_1$ and $\delta(\infty)_1$, $\delta(0)_2$ and $\delta(\infty)_2$ in red knot and as presented by Hobson & Clark (1992) for quail.

For comparison with the performance of these dual-tissue isotopic-clock models, we conducted a similar sensitivity analysis for the single-tissue isotopic-clock model (eqn 2) using quail liver and blood and red knot blood plasma and cells using the same error terms as described before.

STATISTICS

Statistics were conducted using SPSS for Windows 14·0, unless stated otherwise. Data of red knot are presented with SE. To avoid pseudo replication (Evans Ogden, Hobson & Lank 2004), exponential decay curves (eqn 1) were calculated for each individual red knot separately with NONLIN 2·5 (Sherrod 1994; based on the nonlinear least-squares algorithm described in Dennis, Gay & Welsch 1981). The parameters of the individual curves were averaged to obtain the general exponential decay curves. For all analyses, the significance level α was set at 0·05.

Results

ISOTOPIC SIGNATURE OF RED KNOT DIETS

Within shellfish, cockles had a lower δ^{13}C than baltic tellins ($-17 \cdot 1 \pm 0 \cdot 4\text{‰}, n = 10$, and $-15 \cdot 0 \pm 0 \cdot 4\text{‰}, n = 13$, respectively; ANOVA $F_{1,21} = 13 \cdot 45, P < 0 \cdot 01$). δ^{13}C was higher in shellfish than in Trouvit ($-20 \cdot 5 \pm 0 \cdot 3\text{‰}, n = 2$; ANOVA $F_{2,22} = 18 \cdot 06, P < 0 \cdot 001$, Bonferroni analysis).

EXPONENTIAL DECAY CURVES FOR BLOOD CELLS AND PLASMA OF RED KNOTS

Body masses of the red knots were relatively high upon capture (159 ± 3 g, $n = 7$), and dropped considerably in the first days in captivity (Fig. 1a). Thereafter, body mass rapidly increased to stabilize around 143 g (overall mean over the period 14–63 days after start of experiment was 143 ± 3 g, $n = 35$). At the end of the experiment, when the spring fuelling period started, body mass increased again.

At capture, δ^{13}C ratios of blood cells and plasma ($\delta^{13}C_{\text{cells}}$ and $\delta^{13}C_{\text{plasma}}$, respectively) fell between the δ^{13}C of cockles and baltic tellins (Fig. 1b), with $\delta^{13}C_{\text{plasma}}$ below $\delta^{13}C_{\text{cells}}$ (paired Student's t-test, $t_5 = 5 \cdot 29, P < 0 \cdot 01$, day 0). After the diet switch, $\delta^{13}C_{\text{plasma}}$ was rapidly depleted towards δ^{13}C
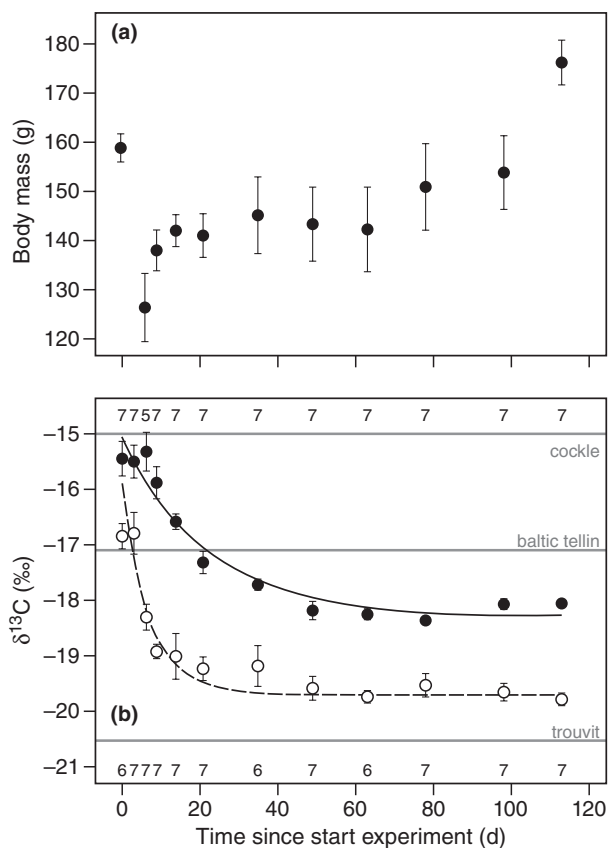
Fig. 1. (a) Variation in mean body mass and (b) mean δ^{13}C in blood cells (closed circles, solid line) and plasma (open circles, dashed line) in red knots with time since start of the experiment. For body mass only cases with stable isotope data available were included. The lines represent the mean of the individual exponential decay curves (see text for equations). Day 0 is day of catching and transport to the NIOZ. Sample sizes for blood cells and plasma are presented in the top and bottom of panel (b), respectively.

of Trouvit. $\delta^{13}C_{cells}$ changed much slower and remained higher than $\delta^{13}C_{plasma}$. Hence, δ^{13}C turnover rates were higher in plasma than in blood cells (0.144 ± 0.028 and 0.046 ± 0.003 day^{-1}, respectively), and half-life was shorter in plasma than in blood cells (6·03 vs. 15·07 days). The average equation for the turnover of δ^{13}C in blood plasma was:

$$\delta(t)_1 = -19.71 - 3.83e^{-0.144t} \qquad \text{(eqn 9)}$$

and for blood cells:

$$\delta(t)_2 = -18.35 - 3.23e^{-0.046t}. \qquad \text{(eqn 10)}$$

COMPARISON OF THE MODELS

Our model and that of Phillips and Eldridge yield different outcomes (Fig. 2, Appendix S1). They also varied in their ability to generate estimates. Whereas our model generally

failed to generate estimates at relatively long periods after the diet switch, Phillips and Eldridge's model was often unable to make a prediction relatively shortly after the diet switch. For those dual isotope measurements where both models generated an estimate of the time since a diet switch, the match between these estimates and the actual time since the diet switch, appeared reasonable, except for the tissue pair muscle and hair in gerbils. However, absolute errors were considerable in many cases (Fig. 2, Appendix S1).

The sensitivity analysis of both models, using dual δ^{13}C measurements in liver and blood in quail (Hobson & Clark 1992) and in blood plasma and cells in red knot, likewise highlights that major differences between the two models may occur despite their fundamental similarity (Fig. 3). Our model showed a satisfying performance up to the moment the half-life time of the slowest turning-over tissue was approached. Around and after this point in time the variation in the estimates increased sharply (Fig. 3, middle panels), not all of the 2000 iterations per time point resulted in an outcome (Fig. 3, top panels), and the median estimated time since diet shift started to deviate from the actual time since a diet switch (Fig. 3, bottom panel). In contrast, Phillips and Eldridge's model performed poorly directly after the diet switch and long after the diet switch, but yielded very satisfying results in the range of *c.* 6–30 days after the diet switch. In this range, most iterations yielded an outcome, the median estimated time since diet shift was close to the actual time since a diet switch, and the variation in the estimates was reasonably low. Still, if knowledge of both the initial and ultimate tissue isotope ratios is available, the use of a single tissue model (eqn 2) yields much more reliable time-since-diet-switch estimates than the two dual-tissue isotopic-clock models (Fig. 3). Predictions up to twice the half-life time of the tissues tended to be very satisfying, with (nearly) all 2000 iterations per time point yielding an outcome and variations tending to be low to at least the half-life time of the respective tissue and often considerably beyond.

Discussion

VALIDITY OF THE MODELS

Our analytical dual-tissue model allows a straightforward calculation of the time since the occurrence of a diet switch using the isotopic ratios in two different tissues measured simultaneously, in combination with knowledge on the turnover rates of these tissues and the actual (i.e. new) diet. Phillips & Eldridge's (2006) calculation procedure is somewhat more cumbersome, as it only allows for the estimation of the time since the diet shift using a numerical routine. But a more important and fundamental difference between the two methods is that Phillips and Eldridge's method not only estimates the time since diet switch but also $\delta(\infty) - \delta(0)$, that is, the difference in isotope ratio between the old and new diets. Within a data set $\delta(\infty) - \delta(0)$ should in principle be held constant as the difference in isotope ratio between the old and new diets cannot vary, but for both the gerbil and the quail data sets,
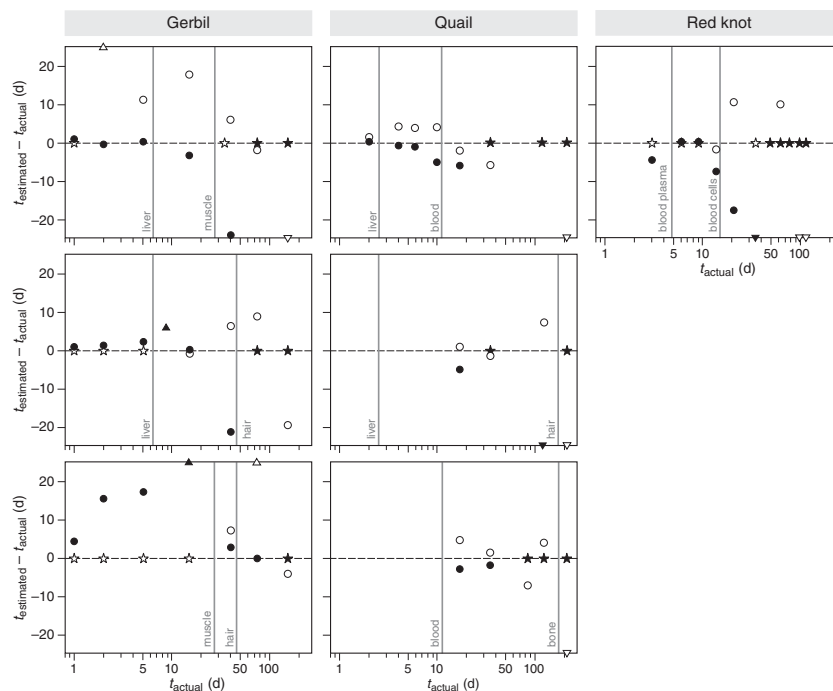
Fig. 2. The difference between estimated time since diet switch ($t_{estimated}$, days) and actual time since diet switch (t_{actual}, days) as a function of the actual time since diet switch for experiments with gerbils (left column panels), quail (middle column panels) and red knot (right panel) using dual $\delta^{13}C$ measurements in different tissues. Estimated time since diet switch was based on the model presented in this study (closed symbols) and that presented by Phillips & Eldridge (2006; open symbols). Differences exceeding −25 or 25 days are indicated by a triangle down or up, respectively; otherwise, circles are used. Sometimes the time since diet switch could not be estimated; this is indicated by a star at the line of no difference (dashed line). Half-life times [= ln(2)/λ] for the tissues of which $\delta^{13}C$ were used in the calculations are depicted along the *X*-axes. Data from Table 1.

Phillips & Eldridge (2006) allowed it to vary for each pair of measurements.

There are more issues to consider when evaluating the usefulness of these models. For instance, the use of the rather simple, first-order exponential isotopic turnover model is based on one-pool kinetics and might only represent a coarse approximation of the real turnover processes (Carleton *et al.* 2008; Martínez del Rio & Anderson-Sprecher 2008; see, however Guelinckx *et al.* 2008). Possibly, the initial delay in the response of the blood cells to the diet shift (Fig. 1b) is an indication of this. Such deviations from the ideal first-order exponential isotopic turnover model need, however, not be detrimental for the isotopic-clock method. They merely result in uncertainty in our parameter estimates and therewith increased inaccuracy in the timing since diet shift, the extent of which needs to be estimated using a sensitivity analysis. The assumption that the difference between $\delta(\infty)$ and $\delta(0)$ for both tissues is identical [i.e. $\delta(\infty)_1 - \delta(0)_1 = \delta(\infty)_2 - \delta(0)_2$], an assumption made by both dual-tissue models, may also not be entirely true (e.g. Hobson & Clark 1992). By definition, all models are simplified representations of the real world and their value resides in their expedience and usefulness despite these simplifications. In many instances, it may be easier to assess the isotopic signature of the new diet rather than the old diet. In those circumstances our model has the advantage over that of Phillips and Eldridge in that no prior knowledge of the tissues under the old diet, prior to the diet switch, is required. Instead, one needs to know the isotopic signature of the new diet. As became clear from both the validation and the sensitivity analysis, relative to the Phillips and Eldridge model ours was particularly good in predicting time since diet switch immediately following the diet switch. There seemed to be a tendency for predictions by the Phillips and Eldridge model to be better when the time since diet switch was close to the half-life time of the slowest turning-over tissue. Neither model was particularly reliable after the half-life time.

In our view the accuracy of both models is more limited than Phillips & Eldridge (2006) suggested for their model, because they did not consider errors in the turnover rates (i.e. λ_1 and λ_2) and only considered the (small!) analytical errors in the measurement of $\delta^{13}C$ neglecting the considerable biological variation that may actually be involved when assessing $\delta(t)_1$, $\delta(t)_2$, $\delta(0)_1$, $\delta(\infty)_1$, $\delta(0)_2$ and $\delta(\infty)_2$. Quite correctly, Phillips & Eldridge (2006) point out that the sensitivity of an isotopic-clock model critically depends on the ratio of the half-life times of the slow and fast tissues used. The sensitivity analysis presented in this study using only two specific combinations of half-life times should thus not uncritically be extrapolated to any combination of half-life times. We rather advocate that whenever researchers want to use our or the Phillips and Eldridge model, they *a priori* work through a sensitivity analysis. In the current absence of a full Bayesian hierarchical model for this problem we suggest to tentatively use the methodology presented here to assess the usefulness of both models to assist in their specific studies.
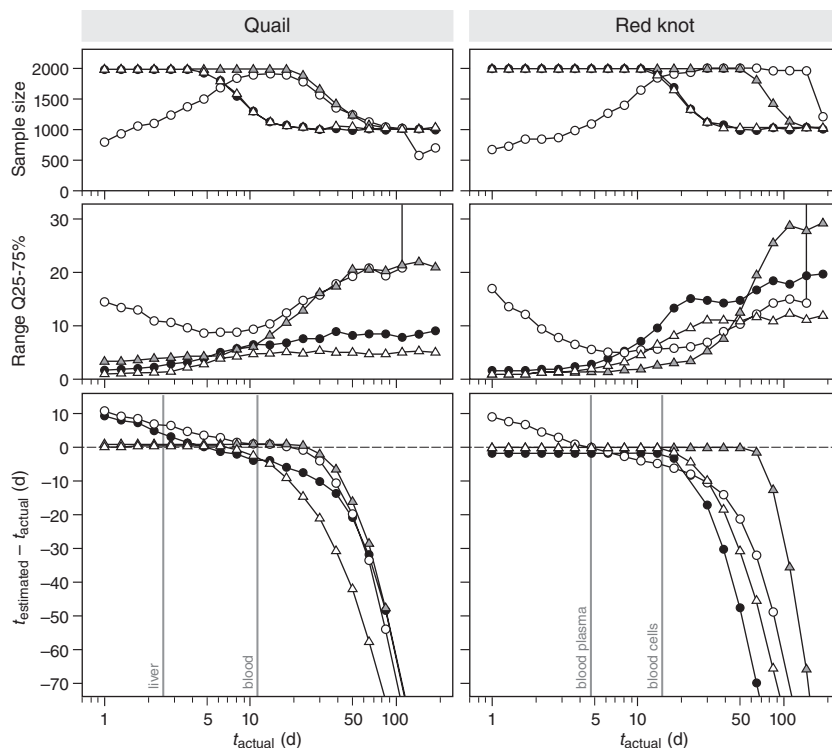
Fig. 3. Results from the sensitivity analysis showing the difference between the median estimated time since diet switch and actual time since diet switch (lower panels: $t_{estimated} - t_{actual}$, days) as a function of the actual time since diet switch (t_{actual}, days) for an experiment with quail using dual $\delta^{13}C$ measurements in liver and blood (left panels, Hobson & Clark 1992) and red knot using dual $\delta^{13}C$ measurements in blood cells and plasma (right panels, this study), taking into account potential errors in measurements and model parameters. Estimated time since diet switch was based on the dual-tissue models presented in this study (eqn 5; closed circles) and by Phillips & Eldridge (2006; open circles) and the single-tissue model (eqn 2) using both the fast turning-over tissues (open triangles) and the slow turning-over tissues (grey triangles). The inaccuracy in these estimates, expressed as the difference between the 75% and 25% quartiles [Q25–75%] are depicted in the middle panels and the sample sizes are depicted in the upper panels. The dashed lines in the bottom panels present no difference ($Y = 0$), whereas the grey lines present the half-lives of the tissues.

Not surprisingly, as it relies on far fewer parameters and thus is less prone to error, the single-tissue model (eqn 2) generally performed better than the two dual-tissue models (Fig. 3). Oppel & Powell (2010) recently evaluated the single-tissue model for a number of bird species using carbon stable isotope ratios in blood with similar partly encouraging results 1–2 weeks after a diet switch. Its application does, however, require more information, that is, both the isotope ratio in the tissue under the old diet, prior to the diet switch, and the stabilized isotopic signature of the same tissue under the new diet, are required. However, using this single-tissue approach it may be easier to accommodate for systems described by two or more compartments (Carleton *et al.* 2008; Martínez del Rio & Anderson-Sprecher 2008) rather than the here assumed one-pool kinetics. Furthermore, in contrast to the dual-tissue model requiring the tissues of interest to be in equilibrium with the diet prior to the shift (see assumption made to derive eqn 5 from eqns 4a and 4b) this is not strictly required for the application of the single-tissue model. One just needs to know exactly the isotope ratio in the tissue of interest prior to the shift. Finally, as for the dual-tissue models, to establish the single-tissue model's suitability for a specific system, we advocate the use of a sensitivity analysis.

PROSPECTS OF BLOOD ISOTOPES AS CLOCK

Using stable isotope data from blood cells and plasma, the time elapsed since the diet switch was better estimated with our dual-tissue model than with the Phillips and Eldridge's dual-tissue model (Figs 2 and 3). Estimates were, however, only valid over a relatively short period, namely up to the half-life of the slowest tissue, blood cells (*c.* 15 days). This interval may prove to be sufficiently long for our purpose; in red knots, the first 2 weeks after arrival in the Wadden Sea are essential for survival, because in this period the gizzard must increase in mass to enable efficient food processing (Dekinga *et al.* 2001; van Gils *et al.* 2003, 2006).

As blood is often easily extracted and contains both a relatively fast and a relatively slow turning-over compartment (i.e. blood plasma and cells, respectively), it makes for an ideal tissue for isotopic-clock work, notably if the turnover times of tissues could be easily obtained from allometric relationships. Therefore, we trawled the literature for data on $\delta^{13}C$ turnover rates in whole blood, blood cells and plasma of birds and mammals (Appendix S2). Unfortunately, the available data is very limited. For mammals we found only data of whole blood $\delta^{13}C$ turnover rates. In

three experiments in bats, turnover rates were considerably lower than in all other species (Voigt *et al.* 2003; Mirón *et al.* 2006). This is most likely related to the low quality of their new diets, which were (very) poor in nitrogen (Mirón *et al.* 2006; Tsahar *et al.* 2008). Therefore, these data were excluded from the analysis. However, its exclusion did not yield a significant relationship between whole blood turnover rate and body mass.

For birds, Bauchinger & McWilliams (2009) recently provided allometric relationships for whole blood and blood cells. Separate allometric relationships explained more of the variance than when data for whole blood and blood cells were combined. The exponents were close to the expected -1/4 (Carleton & Martínez del Rio 2005; -0·29 and -0·27, for whole blood and blood cells, respectively), and the turnover rate of whole blood exceeded that of blood cells. After including our red knot blood cell data the slope of the allometric relationship decreased to a non-significant $-0\cdot20$ ($P = 0\cdot084$, $n = 5$). Also, turnover rates in avian plasma did not yield a significant relationship with body mass.

Whole blood turnover rates in mammals (average 0·03) and birds (average 0·08) were also not significantly different (Student $t_8 = 1\cdot82$, $P = 0\cdot11$). The isotopic-clock models presented here are rather sensitive to variations in tissue turnover rates. For instance, from inspection of eqn 2 it can directly be seen that the estimated time since diet switch is inversely proportional to the turnover rate of the used tissue. If, for instance, the average avian plasma turnover rate was used (0·517) instead of the species-specific red knot value (0·116) this would result in all time estimates being only one-fifth of the true value. The high variability in the turnover rates for blood tissues among species of mammals and birds, as well as the lack of patterns in these rates across taxa and body mass ranges, suggests that using estimates of blood (cells) and plasma turnover derived from allometric relationships for use in isotopic-clock calculations is, as yet, not advisable, at least not for birds and mammals.

Acknowledgements

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Supporting information

The following Supporting information is available for this article:

Appendix S1. Actual time since diet switch and time as estimated by the models of Phillips & Eldridge (2006) and this study.

Appendix S2. Available literature data on average turnover rates of $\delta^{13}C$ in whole blood, blood cells or plasma, and body mass in birds and eutherian mammals.

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