

Estimation of energy expenditure in free-living red deer (*Cervus elaphus*) with the doubly-labelled water method

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Energy expenditure was estimated using the doubly-labelled water (DLW) method in summer in five free-living adult, non-pregnant, non-lactating, red deer (*Cervus elaphus*) hinds (weight 107.3 (SE 0.9) kg; age 6 (SE 1) years) on lowland pasture under typical farming conditions. Climatic conditions were monitored throughout the experiment. Errors due to ^2H losses in CH_4 and faeces were calculated from previous estimates of stoichiometries. CH_4 production, fractionated water loss, urinary N and O_2 consumption were estimated using an iterative approach. The water flux (rH_2O) in these animals consuming only fresh grass was 12 (SE 0.5) kg/d, the CO_2 production (rCO_2) was 1271 (SE 40) litres/d and the mean energy expenditure was 25 (SE 0.8) MJ/d. There were no significant differences in the isotope distribution spaces and flux rates, rH_2O , rCO_2 or energy expenditure using the multi-point or two-point approaches to calculation. The DLW-derived energy expenditure of 25 MJ/d is approximately 20% higher than the recommended intake of 21 MJ/d for adult hinds kept outdoors (Adam, 1986) and, at 757 kJ/kg $^{0.75}$ per d, one third higher than the value of 570 kJ/kg $^{0.75}$ per d for stags penned indoors (Key *et al.* 1984).

Energy expenditure: Doubly-labelled water: Red deer

Energy is often the most important single factor in the nutrition of an animal. When natural diets meet the needs for energy, other essential nutrients (protein, fats, minerals and vitamins) will usually be supplied in amounts at least sufficient for survival (Kay *et al.* 1984). Knowledge of energy expenditure under typical free-living conditions is important when determining levels of feeding in production animals or in assessing the ecological impact of grazing in free-ranging animals. Animals such as red deer (*Cervus elaphus*) typically spend much of their life outdoors, even when farmed, and they may range over large areas. Traditional methods of estimating energy requirements, such as chamber calorimetry or even food-intake studies are therefore of limited use. The doubly-labelled water (DLW) technique (Lifson & McClintock, 1966) is currently the most promising method of estimating energy expenditure in free-living animals. The DLW method has now been successfully validated in ruminant (Fancy *et al.* 1986; Midwood *et al.* 1994) and single-stomached (Haggarty *et al.* 1994a) production animals and may therefore be used with confidence in a wide variety of metabolic states if appropriate corrections are made for the processes which are known to introduce errors into the technique (Midwood *et*

al. 1989; 1993; Haggarty, 1991; Haggarty *et al.* 1994a). In the present study, DLW was used to estimate the energy expenditure of adult non-pregnant red deer hinds in summer whilst on lowland pasture under typical farming conditions.

Energy expenditure may be calculated from DLW data using the two-point or multi-point methods, but the two-point approach has the important advantage for studies in free-ranging animals that the samples of body fluids (typically blood) need only be taken on the first and last days of the labelling period and not each day as with the multi-point approach. Thus, one of the aims of the present study was to compare these two methods of calculation under typical experimental conditions.

In order to calculate water flux (rH_2O) and CO_2 production (rCO_2) it is necessary to correct the isotope flux data for fractionated water loss (Haggarty *et al.* 1988) and any change in pool size (Haggarty *et al.* 1994a) during the course of the labelling period. There are, however, other processes which may introduce errors into the DLW estimate of rCO_2 ; for example, loss of ^2H into products other than water will cause rH_2O to be overestimated and rCO_2 to be underestimated. This may occur during sequestration of ^2H into stable C–H bonds or by exchange of ^2H with labile

Abbreviations: DLW, doubly-labelled water; rCO_2 , CO_2 production; rH_2O , water flux.

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positions on material which is subsequently exported from the body; faeces for example. The sequestration and exchange processes which have the potential to introduce substantial errors into the DLW method in production animals are: fat synthesis, CH₄ production and export of faecal DM (Midwood *et al.* 1989; Haggarty, 1991; Haggarty *et al.* 1994a). The magnitude of these processes must be estimated in order to make the necessary corrections to the isotope flux data (Haggarty, 1991). Under the highly controlled conditions of a validation study it is possible to make direct measurements of faecal losses, CH₄ production and even fat synthesis, but the need to carry out such measurements in a typical DLW study would largely nullify the main advantage of the DLW method which is that it can be used in free-living animals in their natural environment. The final aim of this study was to develop a method of correcting for the processes mentioned without the need to measure them directly, using only the data which are typically obtained in DLW studies in truly free-living animals.

Materials and methods

Animals

Seven adult (age 6.6 (SE 0.5) years), non-pregnant, non-lactating, red deer hinds (weight 103 (SE 2) kg) were studied. Five of the animals were dosed with triply-labelled (²H, ³H, ¹⁸O) water at the beginning of the study period. The two remaining animals were used as controls to quantify any re-uptake of isotope excreted by the labelled animals. Before and during the experiment, the labelled and control animals were maintained together in a lowland field (30 m × 110 m). Animals consumed only the grass in the field and were not provided with any additional feed. The experiment was carried out in the month of August in Aberdeen and climatic conditions were measured in the paddock where the animals were kept.

Samples of grass (approximately 1 kg per sample) were taken from the field on each day of the study and the chemical composition of the pooled sample was analysed. The grass consisted of (g/kg): 590 carbohydrate (polysaccharide equivalents), 150 protein and 64.9 lipid on a dry-weight basis. The energy content of the grass was estimated by bomb calorimetry at 17.389 kJ/g dry weight. The DM content of the grass was 0.1935 g/g wet weight.

At 10.00 hours on the first experimental day a catheter was inserted temporarily in the jugular vein. A blood sample was taken from each of the seven animals to determine the natural abundance of ²H and ¹⁸O in the body water before dosing. The labelled water (approximately 60 ml) was then administered to five of the deer as a sterile saline solution via the catheter. At 15.00 hours a second blood sample (10 ml) was taken and further blood samples were taken daily by vacutainer for the next 10 d from the labelled deer and two control deer. Animals were restrained singly for the administration of dose and blood sampling. Body weight was determined at the beginning and end of the 10 d experimental period. Blood samples were centrifuged to separate the plasma which was then centrifuged through ultramembrane filters (Anachem,

Luton, Beds., UK) to remove any large molecular mass (> 30 000 Da) compounds.

Isotope analysis

Sufficient isotope was administered to raise the ²H and ¹⁸O concentrations in the body water to approximately 160 and 200 parts per million (ppm) excess respectively (0.12 g H₂¹⁸O/kg body weight; 0.16 g ²H₂O/kg body weight) and the ³H to 45 000 disintegrations/min per ml plasma. All stable isotope enrichments were determined on SIRA-10 and SIRA-12 dual-inlet isotope ratio mass spectrometers (VG, Middlewich, Ches., UK) relative to a series of laboratory reference waters previously calibrated against Vienna standard mean ocean water and standard light Antarctic precipitation. The ¹⁸O content of the filtered plasma samples was determined by equilibration with CO₂ in vacutainers (Midwood *et al.* 1992) and ²H by Zn reduction of water (Wong *et al.* 1987) with the modification that 500 mg Zn was used for each reduction. The mean standard error for mass spectrometer analysis was 0.2–0.4 ppm for ²H and 0.1–0.2 ppm for ¹⁸O. The ³H was determined by liquid scintillation counting.

Calculations

Curve fitting was carried out using Maximum Likelihood Program (MLP, Numerical Algorithms Group, Oxford, Oxon, UK). A generalized linear model was fitted to the data with a logarithmic link function and poisson-type error (a heterogeneity factor was calculated from the residual mean deviation) as described elsewhere (Haggarty *et al.* 1994a). The intercepts and rate constants were derived from the fitted parameters. The flux rates, pool sizes, correction for changing pool size and faecal losses and the overall precision of the DLW method were calculated as described previously (Haggarty *et al.* 1994a).

In order to calculate rCO₂ from ²H and ¹⁸O flux rates it is necessary to correct the isotope flux data for fractionated water loss, any change in pool size during the course of the labelling period and loss of ²H into products other than water (Fig. 1). Furthermore, the DLW method only provides an estimate for rCO₂, therefore additional information on O₂ consumption, N loss and, in the case of ruminants, CH₄ production, is required to calculate a value for energy expenditure. In the absence of direct measurements, the estimation of many of the correction factors and additional parameters depends on knowledge of the others before each can be calculated. We have proposed here an approach to the calculation where, after an initial approximation, each parameter is repeatedly calculated until there is no change with further iteration. Initially, the N loss, rH₂O_(methane), rH₂O_(faeces), rO_{2(methane)} and rCO_{2(methane)} were all set to zero and the following values ascribed to fractionation and the RQ: f1 = 0.941, f2 = 0.99, f3 = 1.039, X = 0.1, RQ = 0.94 (estimated from the composition of the diet). The interconnections between the various parameters used in the iteration model are given in Fig. 2. The ¹⁸O and ²H flux rates were held constant whilst the other parameters in the illustration were allowed to vary. All other

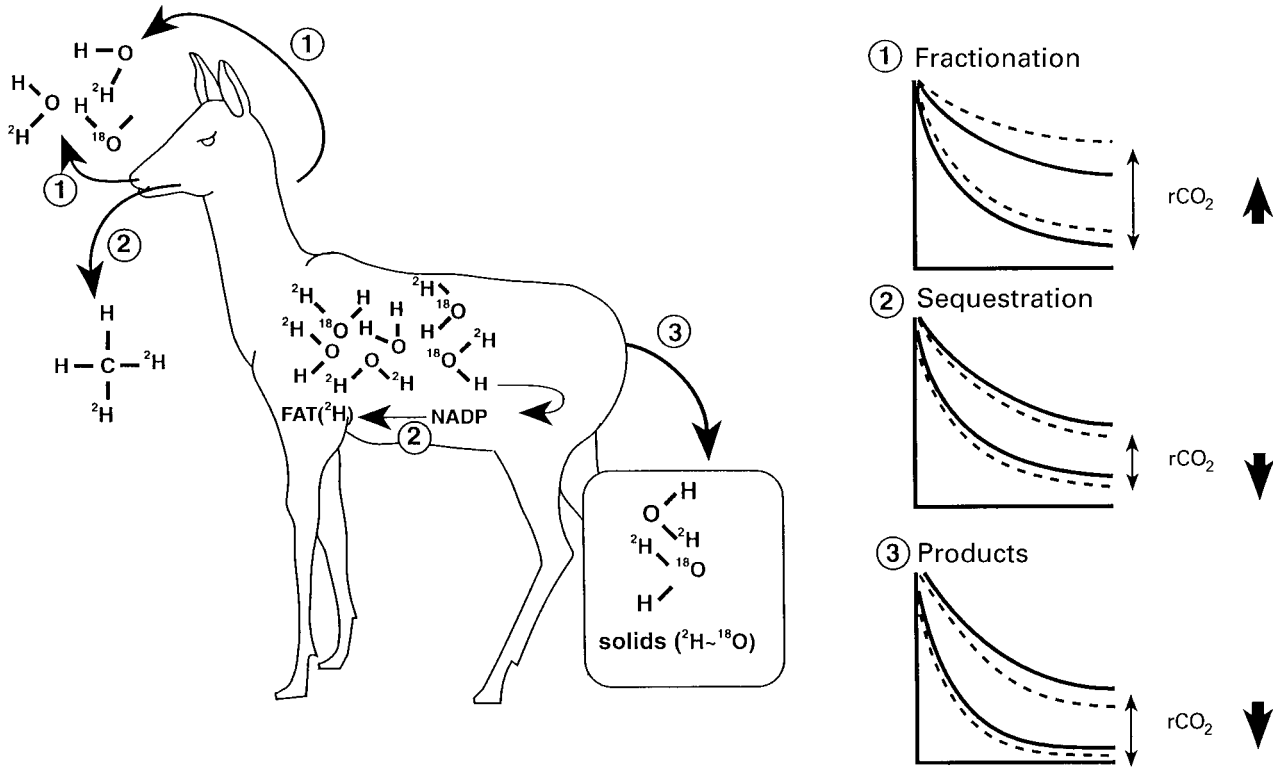


Fig. 1. The main factors which affect the calculation of water flux (rH_2O) and CO_2 production (rCO_2) from 2H and ^{18}O flux rates are: (1) fractionation during evaporative water loss, (2) sequestration of 2H into CH_4 and body fat, (3) loss of 2H and ^{18}O in sequestered and exchangeable positions in faecal solids. The effects of these processes (---) on typical isotope decay curves (—), where the upper line represents 2H and the lower ^{18}O , are shown, together with the effect on the calculation of rCO_2 . Fractionation has a larger effect on 2H than ^{18}O , therefore it is retained longer in the body and the difference in 2H and ^{18}O flux rates is increased, resulting in an overestimate of rCO_2 if not corrected for. Similarly, the rate of sequestration and loss of isotope in products are more pronounced for 2H , resulting in an increased rate of washout and an underestimate of rCO_2 if not corrected for.

parameters and stoichiometries used in the model were held constant. Water flux (rH_2O ; g/d) was calculated from the deuterium flux rate (2H flux; g/d) and water equivalents lost in CH_4 and faeces (g/d):

$$rH_2O = \frac{{}^2H \text{ flux}}{(f1 \times X) + (1 - X)} - (rH_2O_{(\text{methane})} + rH_2O_{(\text{faeces})}). \quad (1)$$

CO_2 production (rCO_2 ; mol/d) was calculated from isotope flux rates (^{18}O flux, 2H flux; mol/d):

$$rCO_2 = \frac{{}^{18}O \text{ flux} - [(f2 \times X \times rH_2O) + (1 - X) \times rH_2O]}{2 \times f3}. \quad (2)$$

CH_4 production (rCH_4 ; litres/d) was calculated from rCO_2 (litres/d) and the ratio $rCH_4:rCO_2$:

$$rCH_4 = rCO_2 \times \text{ratio } CH_4 : CO_2. \quad (3)$$

O_2 consumption (rO_2 ; litres/d) was calculated from rCH_4 and rCO_2 (both litres/d) and the RQ (0.941) estimated from the composition of the diet after correction for the CO_2 equivalents consumed ($rCO_{2(\text{methane})}$; 1 litre/litre rCH_4) and O_2 equivalents produced ($rO_{2(\text{methane})}$; 2 litres/litre rCH_4)

during CH_4 production:

$$rO_2 = \frac{rCO_2 + rCO_{2(\text{methane})}}{RQ} - rO_{2(\text{methane})}. \quad (4)$$

Energy expenditure (EE; kJ/d) was calculated from rO_2 , rCH_4 , rCO_2 (all litres/d) and N loss (g/d):

$$EE = 16.18 \times rO_2 + 5.02 \times rCO_2 - 2.17 \times rCH_4 - 5.99 \times N \text{ loss}. \quad (5)$$

Urinary N loss was calculated from energy expenditure, the digestible energy content of the diet (kJ/g), the digestibility of the diet and the N content of the diet (g/g DM):

$$N \text{ loss} = EE \times \text{digestible energy} \times \text{digestibility} \times N \text{ in diet}. \quad (6)$$

Evaporative water loss (g/d) was calculated from energy expenditure (kJ/d), the proportion of heat lost evaporatively and the latent heat of evaporation of water at 33° (kJ/g):

evaporative water loss

$$= \frac{EE \times \text{proportion of heat lost evaporatively}}{\text{latent heat of evaporation}}. \quad (7)$$

The proportion of water loss which undergoes fractionation

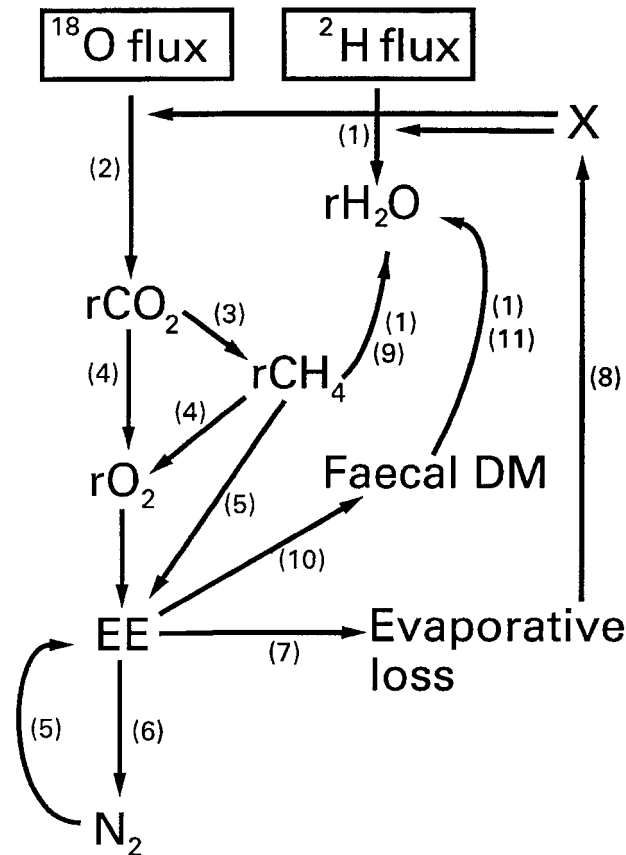


Fig. 2. The interconnections between the various parameters which are allowed to vary in the iteration model are shown here together with the relevant equation number (in brackets) in the text defining the relationship. The only parameters in this illustration which are not allowed to vary are the ^{18}O flux and the ^2H flux. EE, energy expenditure; rCH_4 , CH_4 production; rCO_2 , CO_2 production; rO_2 , O_2 consumption; rH_2O , water flux; X, fractionated water loss.

(X) was calculated from the total evaporative water loss (g) and rH_2O (g):

$$X = \frac{\text{evaporative water loss}}{\text{rH}_2\text{O}}. \quad (8)$$

The water equivalents lost in CH_4 ($\text{rH}_2\text{O}_{(\text{methane})}$; g/d) were calculated from rCH_4 (litres) and the stoichiometry of CH_4 to water loss (g/l):

$$\text{rH}_2\text{O}_{(\text{methane})} = \text{rCH}_4 \times \text{stoichiometry of } \text{CH}_4 \text{ to water loss}. \quad (9)$$

Faecal DM production (g/d) was calculated from energy expenditure, the digestible energy content of the diet (kJ/g DM) and the digestibility of the diet:

$$\text{faecal DM} = (\text{EE} \times \text{digestible energy}) \times (1 - \text{digestibility}). \quad (10)$$

The water equivalents lost in faeces ($\text{rH}_2\text{O}_{(\text{faeces})}$; g/d) were calculated from rCH_4 (litres/d) and the stoichiometry of DM

to water loss:

$$\text{rH}_2\text{O}_{(\text{faeces})} = \text{faecal DM} \times \text{stoichiometry of faecal DM to water loss}. \quad (11)$$

Results and discussion

The average age of the five experimental deer dosed with labelled water was 6 (SE 1) years (Table 1). The age of both the control animals was 8 years. The average body weight of the experimental group was 107 (SE 0.9) kg whilst that of the control animals was 94 kg. During the course of the 10 d experimental period, animals B and C lost weight whilst animals D and E gained weight; there was no change in the body weight of animal A. However, the change in each animal was small and could be accounted for by normal variations in gut fill and the mean weight change was not significant (-0.2 (SE 0.7) kg). It is therefore unlikely that the deer were depositing body fat during the course of the DLW experiment. However, even in the absence of net fat deposition it is still possible that fat turnover could in theory result in water ^2H sequestration. We have previously determined that the sequestration of ^2H into body fat has an insignificant effect on the DLW method in single-stomached and ruminant species in approximate weight balance (Haggarty, 1990; Midwood *et al.* 1993). Therefore, ^2H sequestration into body fat was considered to have a negligible effect on the accuracy of the DLW method in the deer studied here.

During the 10 d DLW period the climatic conditions (Table 2) were typical of the area for the summer month of August.

There was no significant increase in the concentration of ^2H , ^3H or ^{18}O in the body water of the two unlabelled deer sharing the same field with the labelled animals. It was, therefore, assumed that there was no significant re-uptake of isotope from the environment by the labelled animals.

A number of calculation procedures may be used to estimate rH_2O and rCO_2 in a DLW study and may be categorized by the number of data points used. (a) The two-point calculation procedure, originally proposed by Lifson & McClintock (1966) for use in small mammals, relies on two post-dose samples, one taken at the start of the experiment, after the isotope dose has been allowed to equilibrate but before it has been significantly diluted by ingested water (usually 2–9 h after dosing), and another taken at the end of the experimental period; usually two or three biological half-lives. The isotope dilution spaces are calculated from the increase in body water enrichment after equilibration and the rate constants calculated as the gradient between the \log_e of the excess enrichment of the first and last data points. (b) Flux rates may also be calculated using the multi-point approach which involves collecting a series of samples (typically daily) throughout the study. An exponential model is then fitted to the data yielding a value of the extrapolated intercept (used to calculate the isotope distribution space) and rate constant for each isotope (see e.g. Midwood *et al.* 1994). Both the two-point and

Table 1. Details of the red deer used as experimental and control animals

	Experimental deer						Control deer		
	A	B	C	D	E	Mean	SE	G	H
Age (years)	8	7	7	6	4	6	1	8	8
Starting weight (kg)	104.5	109.5	107.5	106.5	108.5	107.3	0.9	94.5	94.0
Weight change (kg/10 d)	0.0	-2.0	-1.5	1.0	1.5	-0.2	0.7		

multi-point approaches also require a sample to be taken immediately before dosing for the estimation of the natural or background abundance of ^2H and ^{18}O in the body water. The results of these two different approaches to estimating the isotope dilution space and body composition are presented in Table 3. There was no difference in the $^2\text{H}:^{18}\text{O}$ dilution space ratio (1.024 (SE 0.004) for the multi-point method and 1.025 (SE 0.004) for the two-point method), or the individual isotope dilution spaces (N^2H , 70.45 (SE 0.81) and N^{18}O , 68.78 (SE 0.91) kg for the multi-point and N^2H , 70.95 (SE 0.65) and N^{18}O , 69.21 (SE 0.83) kg for the two-point method). The isotope dilution spaces may also be used to estimate body water from the relationship between isotope dilution space and body water. The derived estimates of body water were 68.10 (SE 0.90) kg for the multi-point method and 68.53 (SE 0.83) kg for the two-point method; again these values were not significantly different.

Water balance can be used to determine total evaporative water loss, but this will usually overestimate fractionated water loss in sweating species because it contains some evaporated but unfractionated sweat loss (Haggarty *et al.* 1988). However, Johnson *et al.* (1972), who measured sweat gland function in the red deer, found rates of water loss at an ambient temperature of 20° which were within the range observed in sheep and human subjects with a congenital absence of sweat glands. These authors concluded that even at 20° , a temperature exceeding the maximum of 16° achieved during this free-living study, it is unlikely that red deer lose water by active sweating. Under these circumstances total evaporative water loss may be used as an index of fractionated water loss. Brockway & Maloiy (1967) found that at 18° approximately 50% of the heat loss from red deer was by evaporation. The latent heat of evaporation of water at 33° is 2418 J/g (Blaxter, 1989), therefore water loss can be estimated from energy expenditure. However, a problem arises because the value for fractionated water loss is required to calculate rCO_2 and energy expenditure. This circular problem can be overcome using an iterative approach (Haggarty *et al.* 1994b) whereby the raw isotope flux rates (uncorrected for fractionated water loss) are used

to provide an initial estimate of rCO_2 and the fractionated water loss. The energy expenditure can then be used to calculate the proportion of water loss undergoing fractionation (X) and hence a revised estimate of energy expenditure and so on until there is no change with further iterations. Using this approach, we estimated the fractionated water loss, as a proportion of the total water loss, to be 0.42 (SE 0.02) (Table 4). This general approach may also be applied to other correction factors where there is enough information to estimate the parameter of interest from the basic data generated by the DLW method; i.e. rCO_2 and rH_2O .

The stoichiometry of ^2H loss in the exchangeable and sequestered positions in sheep faeces has been estimated at 0.12 g water equivalent per g faecal DM (Midwood *et al.* 1993). The faecal DM production can therefore be estimated from the DM intake and the digestibility of the diet. However, in order to calculate the DM intake it is necessary to know the composition of the diet and the level of energy expenditure. The assumption underpinning this approach is that the animals are in approximate energy balance. This seems reasonable given that there was no significant change in the mean body weight of the deer studied. This problem is similar to that for estimating X and can be approached in the same way. The metabolizable energy provided by the grass was estimated at 10.22 MJ/kg DM and the faecal DM production, measured in sheep consuming the same diet, at 0.41 g faecal DM/g DM intake. The total faecal DM production estimated this way, for the two-point flux data for example, was 965 (SE 47) g/d (Table 4).

It is known that some of the H in CH_4 is derived from body water during methanogenesis (Czerkawski & Breckenridge, 1974; Czerkawski, 1975), therefore CH_4 production provides a further route whereby the deuterium of body water may leave the body, leading to an overestimation of rH_2O and an underestimation of rCO_2 . To calculate the effect of methanogenesis on the DLW method it is necessary to know the rate of CH_4 production (rCH_4) and the stoichiometry of water H incorporation into CH_4 . Midwood *et al.* (1989) have shown that each litre of CH_4 produced will cause rH_2O to be overestimated by 1.052 g and rCO_2 to be underestimated by 0.629 litres. These authors suggested that rCH_4 may be calculated from an estimate of rCO_2 since the $\text{rCO}_2:\text{rCH}_4$ ratio is relatively stable, falling between 10 and 20 for a wide range of animals fed at or above maintenance on very different diets with forage:concentrate ratios of 100:0 through to 20:80 (Midwood *et al.* 1989). For example, Brockway & Maloiy (1967) measured an approximate $\text{rCO}_2:\text{rCH}_4$ ratio of 16 in red deer consuming chopped hay. The exact ratio is mainly determined by the type of diet and level of intake and Midwood (1990) recorded a value of 13 in sheep consuming the same grass as that available to

Table 2. Average environmental conditions during the doubly-labelled water study

Wind speed (knots)	8.7
Rainfall (mm/d)	3.3
Sunshine (h/d)	4.5
Maximum temperature ($^\circ$)	16.6
Minimum temperature ($^\circ$)	9.5
Relative humidity (%)	85

Table 3. Isotope distribution spaces and body composition in five red deer calculated by the multi-point and two-point methods (Individual values for five deer, with mean values and standard errors)

Red deer...	A	B	C	D	E	Mean	SE
Multi-point method							
N ² H (kg)	69.49	73.43	68.66	70.19	70.49	70.45	0.81
SE	1.02	1.46	1.76	0.98	1.13	1.27	0.15
N ¹⁸ O (kg)	67.27	71.60	66.54	68.66	69.84	68.78	0.91
SE	1.03	1.60	1.97	0.97	1.19	1.35	0.19
Ratio N ² H:N ¹⁸ O	1.033	1.026	1.032	1.022	1.009	1.024	0.004
Isotopically estimated body water* (kg)	66.60	70.89	65.88	67.98	69.15	68.10	0.90
Two-point method							
N ² H (kg)	71.04	72.07	68.61	70.76	72.26	70.95	0.65
SE	0.58	0.56	0.53	0.65	0.58	0.58	0.02
N ¹⁸ O (kg)	68.97	70.45	66.19	69.48	70.98	69.21	0.83
SE	0.51	0.52	0.49	0.53	0.52	0.51	0.01
Ratio N ² H:N ¹⁸ O	1.030	1.023	1.037	1.018	1.018	1.025	0.004
Isotopically estimated body water* (kg)	68.29	69.75	65.53	68.79	70.28	68.53	0.83

N¹⁸O and N²H, isotope dilution spaces for ¹⁸O and ²H respectively.

*Body water = N¹⁸O/1.01.

the deer in the present study. If we assume the same value of 13 for the red deer studied here it is possible to estimate the CH₄ production from the rCO₂ in an analogous way to the iterative procedure employed to derive a value for the fractionated water loss. The CH₄ production so estimated was 93 (SE 5) litres/d (Table 4).

Energy expenditure in ruminant animals may be calculated from gas exchanges using the Brouwer equation (see Blaxter, 1989). However, this equation requires knowledge of O₂ consumption (rO₂), rCH₄ and N loss in addition to rCO₂. The value for rCH₄ can be derived as described earlier and the N loss assumed to be equal to N intake, calculated from the N and energy contents of the diet and the estimated energy intake in these animals which were essentially weight stable.

With the raw isotope flux data, and the additional information generated, it is possible to calculate rH₂O, rCO₂ and energy expenditure. A comparison of the multi-point and two-point methods showed no significant difference in any of these parameters (Table 5). For the multi-point approach the values were: rH₂O, 11 970 (SE 543) g/d; rCO₂, 1271 (SE 40) litres/d; energy expenditure, 25.23 (SE 0.80) MJ/d. For

the two-point approach the values were: rH₂O, 11 955 (SE 723) g/d; rCO₂, 1211 (SE 59) litres/d; energy expenditure, 24.05 (SE 1.18) MJ/d. None of the differences was statistically significant.

The animals in the present study were given two isotopes of H (the stable isotope ²H and the radio-isotope ³H) in order to calculate fractionated evaporative water loss as described elsewhere (Haggarty *et al.* 1988). The rationale behind this approach is that the isotope fractionation which occurs during evaporation is more pronounced for ³H than ²H, therefore the difference in flux rates for ³H and ²H can be used to estimate the proportion of water loss which has undergone fractionation. However, two problems arise when using this approach. The first is that the difference between the ³H and ²H fluxes resulting from fractionation is relatively small. Taking the measured ²H flux and an estimate of the expected ³H flux for the fractionated water loss calculated by the iterative approach (X 0.42), the difference between ³H and ²H flux would be approximately 95 g/d (Table 6). The high value for rH₂O in these animals (almost 12 kg/d) and the correspondingly high standard error on the flux rate estimates (100 g/d for ²H, which

Table 4. Factors affecting the accuracy of the doubly-labelled water method for estimating energy expenditure in red deer* (Individual values for five deer, with mean values and standard errors)

Red deer...	A	B	C	D	E	Mean	SE
Fractionated water loss (X)	0.41	0.43	0.45	0.46	0.34	0.42	0.02
CH ₄ production (litres/d)	110	87	89	95	84	93	5
Effect on rH ₂ O (g/d)	116	92	94	100	89	98	5
Effect on rCO ₂ (litres/d)	-69	-55	-56	-60	-53	-58	3
Faecal DM (g/d)	1140	905	921	984	874	965	47
Effect on rH ₂ O (g/d)	148	118	120	128	114	125	6
Effect on rCO ₂ (litres/d)	-88	-70	-71	-76	-68	-75	4
Body-weight change (g/d)	0	-200	-150	100	150	-20	68
Effect on rH ₂ O (g/d)	0	58	56	-33	-66	3	24
Effect on rCO ₂ (litres/d)	0	18	14	-9	-14	2	6

rH₂O, water flux; rCO₂, CO₂ production.

*Based on flux rates derived from two-point data. Apart from fractionated water loss, the main factors affecting the doubly-labelled water-derived flux rate estimates in the deer studied here were CH₄ production, faecal DM production and the change in body water associated with the small fluctuations in body weight. These are given here together with their effects on rH₂O and rCO₂.

Table 5. Water flux, CO₂ production and energy expenditure in red deer calculated by multi-point and two-point methods (Individual values for five deer, with means and standard errors)

Red deer...	A	B	C	D	E	Mean	SE
Multi-point method							
Water flux (g/d)	13800	11006	11332	11087	12628	11970	543
SE	174	203	332	150	189	210	32
CO ₂ production (litres/d)	1415	1238	1177	1234	1290	1271	40
SE	58	61	104	59	81	72	9
Energy expenditure (MJ/d)	28.09	24.58	23.36	24.49	25.60	25.23	0.80
SE	1.30	1.38	2.34	1.32	1.82	1.63	0.20
Two-point method							
Water flux (g/d)	14249	10755	10619	11093	13059	11955	723
SE	116	84	83	105	106	99	7
CO ₂ production (litres/d)	1432	1136	1157	1236	1097	1211	59
SE	104	77	77	90	95	89	5
Energy expenditure (MJ/d)	28.42	22.56	22.96	24.53	21.78	24.05	1.18
SE	2.35	1.74	1.74	2.03	2.14	2.00	0.12
Difference							
Energy expenditure (%)	1.18	-8.24	-1.71	0.17	-14.94	-4.71	3.04

is measured by high precision mass spectrometry, and twice that (200 g/d) for ³H which is measured by scintillation counting with a lower precision) means that an expected difference of 95 g/d could not be detected with this method at these water flux rates. Furthermore, ³H and ²H will undergo different degrees of fractionation during incorporation into CH₄ and faeces, for example, but the isotope effects in these processes have not been quantified. Therefore, when there is significant production of CH₄ and faecal DM, as in ruminant animals, the methodology may be unreliable. For example, the additional water fluxes associated with CH₄ and faeces production are 98 and 125 g/d respectively. Therefore, a significant isotope discrimination effect between ³H and ²H in these processes would be enough to obscure the expected difference between ³H and ²H flux rates in these animals. Although the mean triply-labelled water derived value for X (0.6) was close to the iteratively derived value of 0.42, the standard error was extremely high (2.04), primarily due to the poor precision of the H flux rate estimates. Thus, the value for X derived by iteration has been used here to calculate energy expenditure.

The calculation of energy expenditure from DLW data, as described here, requires the use of a number of

stoichiometries and relationships derived in other studies and other species, therefore it is important to determine the sensitivity of the calculation to errors in these parameters. The results of a series of calculations of the percentage change in energy expenditure with changes in each of the parameters of interest are presented for deer A in Table 7. The value for each parameter in deer A, derived as described earlier, is assumed to produce the correct value (0 % error on energy expenditure). The percentage error in the calculated energy expenditure was determined for a range of values; it is worth noting that the relationship between energy expenditure and the parameters of interest was linear for all those studied with the exception of the rCO₂:rCH₄ ratio which produces a hyperbolic relationship when plotted against the error on energy expenditure. While the calculation of energy expenditure is relatively sensitive to the absolute amount of CH₄ produced it is relatively insensitive to the rCO₂:rCH₄ ratio within the physiological range. Taking a range of values for the rCO₂:rCH₄ ratio, 10 through to 16, which should cover most ruminant animals, the error on energy expenditure only ranged from -0.41 to +0.24 %. The calculation of energy expenditure is also relatively insensitive to realistic variations in the digestibility of the diet and N loss. It is

Table 6. Calculation of fractionated water loss (X) in red deer from ³H and ²H flux rates (Individual values for five red deer, with mean values and standard errors)

Red deer...	A	B	C	D	E	Mean	SE
N ³ H (kg)	71.73	71.11	76.43	64.20	54.85	67.66	3.75
Ratio N ² H:N ³ H	0.990	1.014	0.898	1.102	1.317	1.064	0.071
N ³ H flux (g/d)	14044	10262	11474	10126	11560	11493	703
SE	332	184	276	130	94	203	44
N ² H flux (g/d)	14159	10628	10492	11047	13056	11876	734
SE	116	84	83	104	106	99	6
X calculation from ² H and ³ H fluxes	0.42	1.65	-7.05	3.51	4.48	0.60	2.04
Calculation of expected ³ H flux using X derived from iteration approach							
Expected ³ H flux for iterative calculation of X	14046	10539	10401	10949	12970	11781	731
Expected difference (² H- ³ H flux, g/d)	113	89	91	98	86	95	5

N²H and N³H, isotope dilution spaces for ²H and ³H respectively; X, fractionated water loss as a proportion of total water loss.

Table 7. Sensitivity of energy expenditure to assumed stoichiometries, calculated using the doubly-labelled water-derived flux rates from animal A

	Minimum	Assumed	Maximum
RQ	0.900	0.941	1.000
Error on energy expenditure (%)	3.78	0.00	-4.77
Fractionated water loss (X)	0.10	0.41	0.50
Error on energy expenditure (%)	2.37	0.00	-0.62
rCH ₄ (litres/d)	50	110	150
Error on energy expenditure (%)	3.02	0.00	-1.89
Ratio rCO ₂ :rCH ₄	10	13	16
Error on energy expenditure (%)*	-0.41	0.00	0.24
Digestibility of diet	0.50	0.59	0.60
Error on energy expenditure (%)	2.05	0.00	-0.18
N loss (g/d)	200	245	300
Error on energy expenditure (%)	0.97	0.00	-1.09
Specific heat capacity of water (J/g)		2.418	2.447
Error on energy expenditure (%)		0.00	0.14

rCH₄, CH₄ production; rCO₂, CO₂ production.

*Non-linear relationship between the ratio rCO₂:rCH₄ and energy expenditure.

slightly more sensitive to fractionated water loss although again, in this case, it is perhaps the energy required to evaporate water which is most relevant to a realistic estimate of the likely error. It can be seen that changing the value from that for evaporation at 33° (2.447 kJ/g) to that at 20° (2.418 kJ/g) (Blaxter, 1989) has a negligible effect on energy expenditure. Probably the single most important parameter in modulating energy expenditure is the RQ as a relatively small change from 0.900 to 1.000 can change the estimate of energy expenditure by almost 9%. Estimation of the RQ is particularly problematic in animals which are growing or mobilizing body tissue as the RQ cannot simply be derived from the composition of the diet, it must also be adjusted for the composition of tissue gained or lost.

In this model the only parameters which were not derived but measured directly were the ²H flux and ¹⁸O flux. The animals studied were assumed to be in energy and material balance, therefore the RQ was obtained from the composition of the diet. For the derived parameters, it was necessary to know the previously measured stoichiometries and to set all the derived parameters either to zero or some initial approximation in the first cycle of iteration. However, even when some of the unknown parameters were initially set to zero, the convergence to the correct values for rH₂O, rCO₂ and energy expenditure was very rapid; within only two iterations the values for rH₂O, rCO₂ and energy expenditure had converged to within 0.05% of the value after ten iterations.

Failure to correct for sequestration would have resulted in a 5% underestimate because of CH₄ production and a 6% underestimate because of faecal DM loss. Since these are cumulative the resulting error on the calculated energy expenditure would be -11%. However, it is already well established that it is necessary to correct for these processes, the issue is how well this can be done and the uncertainty which remains because of a lack of primary data. We have tried to estimate the parameters using an iterative approach and have evaluated the likely uncertainty on the calculation of CO₂ production because of imprecise information on stoichiometries etc. (Table 7). Given realistic estimates of these parameters we expect the greatest uncertainty to come

from the estimate of RQ then CH₄ production then fractionated water loss, with likely uncertainties in the other parameters accounting for only a few percent. The choice of parameters to include in the iteration procedure largely depends on how good the initial estimates of unmeasured parameters are. Thus, the results in Table 7 indicate that the initial estimates of parameters such as the rCO₂:rCH₄ ratio, or the digestibility of the diet, or the specific heat capacity of water were sufficiently good that they could have been fixed, and therefore removed from the iteration process, without much loss of accuracy. However, care needs to be taken that inconsistencies are not introduced by fixing parameters which should be allowed to vary when setting up this type of model. For example, the N loss will depend on the energy intake and hence energy expenditure and therefore should be allowed to vary with energy expenditure.

Conclusions

The energy requirements of deer at pasture cannot be readily predicted from measurements made indoors because of the additional demands of muscular activity and thermoregulatory thermogenesis in free-living animals. An illustration of the effect of such additional demands can be seen by comparing the feeding studies of Fennessy *et al.* (1980), who estimated the energy requirement of red deer stags fed outdoors in winter at 850 kJ/kg^{0.75} per d, with feeding studies of red deer stags penned indoors (Key *et al.* 1984) where the estimated energy requirement was 570 kJ/kg^{0.75} per d. Unfortunately, outdoor feeding studies of this type are difficult to carry out, as indicated by the small number of such reports, and they inevitably interfere with normal behaviour such as foraging. It has, therefore, been necessary to develop alternative approaches to estimating energy expenditure in free-living animals and the most promising of these is the DLW method (Lifson & McClintock, 1996). However, this method only provides an estimate for rCO₂, therefore additional information on O₂ consumption, N loss and, in the case of ruminants, CH₄ production, is required to calculate a value for energy expenditure. We have proposed here an approach to calculation where, after an initial approximation, each parameter is repeatedly calculated until there is no change with further iteration. In practice, the convergence to stable values occurs within only a few cycles of iteration and the accuracy of the final estimates depends only on the accuracy of the stoichiometries of the relationships between the parameters of interest and knowledge of external factors such as the composition of the diet. Analysis of the sensitivity of this approach to errors in the parameters and stoichiometries indicates that, with the exception of the RQ, the approach is relatively insensitive to likely physiological variations in the parameters used.

The two approaches to sampling used in DLW studies, multi-point and two-point were compared. The animals studied here were accustomed to handling and could be sampled relatively easily every day. However, this would not be the case in animals ranging freely over rough pasture. Furthermore, daily capture and sampling could, in itself, significantly affect the energy expenditure of free-ranging animals. In such animals, the two-point methodology, where

body fluids are sampled only at the beginning and end of an experiment, typically lasting between 1 and 2 weeks, would be a more feasible approach. A comparison of multi-point and two-point calculations showed that there was no significant difference in the energy expenditure derived by these approaches in the animals studied here. Apart from the advantage of minimal interference with normal behaviour, the two-point method also has the advantage that the estimate of precision does not include a term for daily variation in rH_2O and rCO_2 . Such daily variation is not usually of interest in DLW studies but the multi-point approach necessarily includes this term in the equivalent estimate of precision.

The estimate of precision on the value for energy expenditure for each animal is useful for between-animal comparisons. For example, it allows us to identify that the oldest animal studied here had a significantly higher level of expenditure than the others despite having the lowest body weight. Since all the animals were exposed to the same mild climate, this difference was presumably due to a higher physical activity, possibly reflecting the dominance of this hind within the social group. No study was made of social interactions in these animals but this observation illustrates how the DLW method may provide information in addition to the estimation of energy requirements of groups.

The practical advantages afforded by the two-point method over the multi-point method make this by far the easiest method to use and the only feasible method for use in truly free ranging animals. Given that there was no significant difference in the results obtained using these two approaches it would appear that the two-point methodology may be used with confidence in such animals. The main advantage of the multi-point method is that the possibility of bias due to an unreliable sample or analysis is minimized by the averaging effect of large numbers of samples. This is not so important that it would over-ride the advantages of the two-point methodology in free-ranging animals but, where both approaches have been used, the multi-point method is likely to produce the better estimate of energy expenditure. Using the data from the multi-point calculation we estimate that the energy expenditure of the red deer studied here (mean body weight 107 kg) was 25 MJ/d. This is 20% higher than the value of 21 MJ/d recommended for adult red deer hinds kept outdoors (Adam, 1986) and, at 757 kJ/kg^{0.75} per d, a third higher than the value of 570 kJ/kg^{0.75} per d for stags penned indoors (Key *et al.* 1984). These values for energy expenditure are probably realistic for hinds in typical lowland farming conditions in summer. Energy expenditure in winter may be higher because of the physical activity associated with foraging over rough terrain or it may be reduced because of reduced diet-induced thermogenesis associated with reduced food intake and an adaptive reduction in physical activity as a strategy to increase the efficiency of utilization of available food. The ambient temperature during the summer experimental period (maximum of 16.6°, minimum of 9.5°, with little wind) should represent a minimal thermal stress to red deer, therefore it seems unlikely that thermoregulatory thermogenesis would account for much of the difference between stags penned indoors and the hinds studied outdoors. That leaves the

energy cost of physical activity. Anderson (1976) has proposed that even in freely-ranging deer, normal movements about the hill would probably increase energy requirements by only 10–20% above those of the stationary animal. However, when compared with the value for stags penned indoors, the results presented here for animals kept in a relatively small, lowland paddock, suggest an increase of about 33%. Where animals have to forage over large areas to obtain sufficient intake of a poorer quality diet the energy cost of physical activity may be considerably higher. Similarly, more extreme weather conditions could further increase the energy requirements of the animals studied here under mild summer conditions. By using the sampling and methodological approaches described here, the DLW method could provide estimates of the energy requirements of red deer under more extreme conditions.

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