Loss of deuterium in faecal solids and by sequestration in reindeer: effect on doubly labelled water studies

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Abstract: An underlying assumption when estimating total energy expenditure (TEE) using doubly labelled water (DLW) is that the injected isotopes (^{18}O and ^{2}H) leave the body only in the form of CO_2 and H_2O. However, both isotopes have additional routes of loss. We quantified the loss of ^{2}H (i) attached to faecal solids and (ii) by sequestration into newly synthesised fat in reindeer (*Rangifer tarandus tarandus*). Estimates of the errors caused by these processes were applied to data from DLW studies with reindeer in summer and in winter. Given the net rate of faecal dry matter output and lipid synthesis in the present study, ignoring both sources of error caused the TEE of reindeer to be underestimated by approximately 5% in winter and approximately 9% in summer. The separate effect of each source of error was evaluated in summer. If ignored, loss of ^{2}H through sequestration alone caused TEE to be underestimated by approximately 3.7%. Similarly, if ignored, loss of ^{2}H attached to faecal solids alone caused TEE to be underestimated by approximately 5.9%.

Key words: cervid, energy expenditure, oxygen-18, *Rangifer,* seasonal physiology.

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Introduction

The doubly labelled water (DLW) technique (Lifson *et al.,* 1955) for the measurement of carbon dioxide production rate (r_{CO2}) and the estimation of total energy expenditure (TEE) has been applied in a wide variety of species of birds and mammals (e.g. Nagy, 1987; 1994). The method is based on injection and subsequent measurement of the rate of disappearance of the oxygen isotope ^{18}O and a hydrogen isotope (most commonly ^{2}H, although ^{3}H is also used) from the body water pool. The basic assumption underlying this method is that ^{2}H is washed out as water whilst ^{18}O is lost as both water and CO_2. The amount of CO_2 produced by the animal during the experiment can then be calculated from the difference in the rates of disappearance of the two isotopes. TEE in the same period can be calculated from the volume of CO_2 produced provided the respiratory quotient (RQ) is known. The successful application of the DLW method rests on the assumption that the isotopes leave the body only as H_2O and CO_2. However, ^{2}H atoms in particular can also be lost from the body water pool by exchanging with ^{1}H in labile positions on other molecules which are subsequently exported from the body (e.g. milk solids, faecal solids) and by sequestration into *de novo* synthesised molecules.

Notwithstanding its widespread application in physiological research, particularly in humans, the DLW method has been applied in only six species of ruminants (Fancy *et al.*, 1986; Nagy *et al.*, 1990; Parker *et al.*, 1990; Midwood *et al.*, 1994; Nagy & Knight, 1994; Gotaas *et al.*, 1997; Haggarty *et al.*, 1998). The paucity of DLW studies in ruminants can be attributed to uncertainty of (i) the effect of the large quantity of water in the gastrointestinal tract relative to the total body mass on the rate of equilibration of isotopically labelled water, (ii) the loss of ^2H in methane produced by microbial fermentation in the rumen, (iii) the loss of ^2H in faecal solids which might be of greater importance in ruminants than in monogastric animals because ruminants produce more faeces, and (iv) the sequestration of ^2H in *de novo* synthesised tissue, especially fat (e.g. Fancy *et al.*, 1986; Midwood *et al.*, 1993).

In reindeer, ^3H_2O injected either intravenously or intraruminally is fully equilibrated within 8 h (Gotaas & Tyler, 1995). The loss of ^2H in the methane produced by these animals results in an underestimation of the rate of production of CO_2 (r_{CO_2}) measured by the DLW method of less than 3% (Gotaas & Tyler, unpubl.) compared to 6.5% in sheep (Midwood *et al.*, 1989). The present study examined the remaining issues of non-water losses of ^2H in reindeer (*Rangifer tarandus tarandus*) (i) in labile positions on faecal solids ('exchangeable loss') and (ii) by sequestration into *de novo* synthesised fat. The effect of these processes on the DLW method were assessed by relating the results to data from two DLW studies in reindeer, one with four adult captive female animals in summer and in winter (Gotaas *et al.*, 1997) and one with three young (22 months) free-living male animals in winter (Gotaas *et al.*, in press).

Methods

Loss of ^2H in faecal solids

Experimental trials

Trials were carried out in three adult (>4 years old) non-pregnant, non-lactating female Norwegian reindeer [A, B and C, mean body mass (BM) 86.2 (range 79.0 - 91.0) kg in summer and 82.0 (range 78.0 - 85.0) kg in winter] which had been accustomed to handling over several years. The study consisted of one summer experiment (conducted between 29th June and 13th July 1993) and one winter experiment (conducted between 11th January and 8th February 1994). Prior to and between experiments the animals were kept together in an outdoor enclosure (approximately 2700 m^2) at the Department of Arctic Biology, University of Tromsø (69°40'N), where they had access to natural vegetation including birch (*Betula pubescens*), willows (*Salix* spp.) and sedges (*Carex* spp.) and were also provided with a commercially available pelleted ration, RF-80 (Bøe & Jacobsen, 1981) and water or snow *ad lib.* One week before and throughout each trial they were kept in individual semi-outdoors gravelled paddocks (area approximately 120 m^2) with *ad lib.* access to the pelleted ration and water or snow.

At each trial the animals were weighed to 0.1 kg on an electronic balance (Alpha-100, Farmer Tronic, Denmark) and then injected with sufficient DLW (approximately 90 g; enrichment of ^2H_2O and $H_2{}^{18}O$ of approximately 10%) to enrich body water to approximately 160 ppm excess. A 50 cm silicone tube leading from a 50 ml syringe containing physiological saline was connected to a catheter inserted into the left jugular vein. Pyrogen-free DLW made isotonic by adding 0.9% w/v NaCl was injected from a syringe into the lumen of the tube which was flushed with saline before the needle was withdrawn, thus ensuring complete administration of the isotopes. The exact amount of DLW injected was determined gravimetrically by weighing the dose syringe with its needle to 0.001 g before and after injection.

Between three and six days after dosing with DLW, samples of faeces (approximately 25 g wet weight per sample) were taken from the rectum of each reindeer, put immediately into screw-cap plastic vials and stored at -20 °C.

Exchangeable ^2H

Four aliquots from each sample of faeces (each aliquot approximately 1.5 g wet weight, weight recorded to 0.0001 g) were placed in separate tubes and freeze-dried by vacuum sublimation using the method of Midwood (1990). The extracted water was combined by aliquot and stored at -20 °C prior to determination of ^2H. The remaining dry matter from each aliquot was then placed in a screw-cap vial to which was added 3 g of unenriched tap water, the exact amount and initial enrichment of ^2H of which was known. The vials were placed in a sonic water bath (Branson B2200 E4, U.S.A.) for three hours followed by a shaking water bath at 37 °C for 21 h to ensure thorough mixing of the dry

matter and the wash-water. The wash-water was then removed by vacuum sublimation, combined by aliquot and stored at -20 °C for determination of ^2H.

Calculation of apparent water flux due to loss of ^2H in faecal solids

For each animal, we determined the apparent water flux due to loss of ^2H in faecal solids (expressed as g $H_2O \cdot g^{-1}$ faeces dry matter (DM)) according to Midwood *et al.* (1993):

$$\frac{gH_2O}{g\ \text{faeces DM}} = \frac{mol^2H \cdot g^{-1}\ \text{faeces DM}}{mol^2H \cdot g^{-1}\ \text{body water}} \qquad \text{(equation 1)}$$

In equation 1, mol ^2H per gram faeces DM is determined by:

$$mol^2H \cdot g^{-1}\ \text{faeces DM} = \frac{g\ \text{wash-water}}{18.0152} \cdot \frac{\text{wash-water final enrichment} \cdot 10^{-6}}{g\ \text{faeces DM}}$$

(equation 2)

where 'g wash-water' is the amount of wash-water added to the dry faeces, 18.0152 is the molecular weight of H_2O, 'wash-water final enrichment' is the ^2H_2O-enrichment (ppm excess) of the wash-water after resuspension and 'g faeces DM' is the amount of faecal dry matter to which the wash-water was added.

Mol ^2H per gram body water in equation 1 is given by:

$$mol^2H \cdot g^{-1}\ \text{body water} = \frac{\text{body water enrichment} \cdot 10^{-6}}{18.0152}$$

(equation 3)

where 'body water enrichment' is the ^2H_2O-enrichment (ppm excess) of body water relative to the pre-dose level.

Sequestration of ^2H in newly formed fat

Experimental trials

Trials were carried out on five male reindeer (E, F, G, H and I) born in May 1994. The animals were taken to the Department of Arctic Biology from natural mountain pasture in northern Norway in March 1995. At the time of arrival the animals had a mean BM of 31.7 (s = 3.9; range 25.0 - 35.0) kg. The animals were kept together in an outdoor enclosure (approximately 2700 m^2) where they had access to food and snow or water *ad lib.* as described above. After four months (i.e. in July 1995) the animals were moved to individual metabolism crates placed side by side in a temperature controlled room (temperature 5 to 10 °C) where they were exposed to simulated natural photoperiod and provided with the pelleted ration (RF-80) at approximately 90% of their *ad lib.* level. The animals were weighed to 0.1 kg on an electronic balance approximately once every ten days in the enclosure and once every three days while in the crates.

After three weeks in the crates, the animals were weighed (mean BM 71.0 (s = 5.9; range 61.5 - 77.0) kg and a blood sample (10 ml), from which to assess background levels of ^2H, was collected from each animal via an indwelling catheter in the right jugular vein. Four of the reindeer were then injected with sufficient sterile, pyrogen-free ^2H_2O (approximately 15 ml 99.8% enrichment) to enrich body water to approximately 300 ppm excess ^2H. The ^2H_2O was injected intravenously in the left jugular vein in the manner described above. The control animal was injected intravenously with 15 ml sterile, pyrogen-free physiological saline.

A high level of ^2H was maintained in the body water pool of the four experimental animals to maximise the incorporation of ^2H into *de novo* synthesised fat by providing them with *ad lib.* drinking water with a known enrichment of ^2H (approximately 300 ppm excess) for three weeks following injection of the dose. The control animal was provided with local tap water. Blood samples (20 ml) were collected from all animals once a week by jugular venipuncture using heparinized Vacutainer® tubes (Becton Dickinson Vacutainer Systems Europe, France) and centrifuged (15 min at 1600g) immediately after collection. The plasma was then transferred to Ultrafree-CL® tubes (30 000 NMWL) (Nihon Millipore Ltd., Japan) and centrifuged for 2 h at 4300g in a Sorvall RC5C centrifuge equipped with a Sorvall SS-34 rotor (DuPont Company, Newtown, CT, U.S.A.). The ultrafiltrate was stored in cryo-tubes (Greiner labortechnik, Germany) at -20 °C prior to determination of ^2H.

All five animals were slaughtered three weeks post-injection. The gastrointestinal tract (oesophagus to anus) was emptied, washed with water and homogenised in a mincer (TK 20, Kilia Fleischereimaschinenfabrik, Fritz Reimers Gmbh. u. Co., KG, Kiel, Germany) together with all visceral organs. One half by weight of the homogenate was then ground with one sagittal half of the corresponding empty carcass (consisting of the animal

including head and feet, but excluding skin and fur) in a grinder (Palmia No. 4726, Palmia, Sweden) equipped with a 18 mm mesh sieve. The resulting material was then homogenised in a mincer (TK 20). One kg of homogenate from each animal was freeze dried (Alpha 1-4, Martin Christ Gefrierertrockningsanlagen GmbH, Germany) and ground to powder in a commercial blender (Waring, New Hartford, CT, U.S.A.). Fatty acids (FA) were saponified with 30 ml of 2 M KOH and extracted with diethyl-ether from a 1 g aliquot of the dry powder from each animal following a procedure similar to that described by Haggarty *et al.* (1987) and Midwood *et al.* (1993). After sealed bomb combustion (McGaw *et al.*, 1988) of the extracted FAs, the ^2H_2O-enrichment of the water produced by combustion was determined by mass spectrometry.

Calculation of apparent water flux due to loss of ^2H from sequestration

The apparent water flux due to loss of ^2H by incorporation into newly synthesised lipid was determined by combining a water equivalence factor based on the stoichiometry of ^2H incorporation into FAs, with the estimated rate of FA synthesis for each animal. The water equivalence factor (g $H_2O \cdot g^{-1}$ FA synthesised) was calculated according to Haggarty (1990) from the chemical composition of reindeer adipose tissue (Garton & Duncan, 1971) where the average fatty acid (FA) formula is $C_{17.298}H_{33.707}O_2$ (mol wt 273.737 amu, triacylglycerol (TGA) mol wt 859.259 amu). Jungas (1968) showed that 53% of the hydrogen of synthesised FAs was derived from water. Accordingly, if all FAs in a sample are *de novo* synthesised, the ^2H enrichment in the FAs will be 53% of that in the precursor body water labelled with ^2H. Hence, the synthesis of one mole of FA in a reindeer will result in the incorporation of 17.865 moles of ^2H (33.707 · 0.53) and the deposition of one mole of TGA will result in the sequestration of 53.594 moles of ^2H, equivalent to 62.372 millimoles ^2H per gram fat (53.594/859.259 · 1000). In terms of apparent water flux this corresponds to 0.5618 g $H_2O \cdot g^{-1}$ fat synthesised.

The net rate of FA synthesis during the entire experimental period was estimated according to Haggarty *et al.* (1991):

$$\text{FA synthesis (mol)} = \frac{\text{combustion water enrichment}}{\text{body water enrichment} \cdot 0.53} \cdot \text{Total FA} \quad \text{(equation 4)}$$

where the enrichment of both the water produced by combustion of FA and the body (precursor) water is given in ppm excess. The total FA content of the carcass is given by:

$$\text{Total FA (mol)} = 3 \cdot \frac{\text{weight of lipid (g)}}{\text{mol wight of lipid (amu)}} \quad \text{(equation 5)}$$

where the weight of lipid in the carcass is given in grams and the mol weight of reindeer lipid expressed in gram · mol^{-1} is calculated from data published by Garton *et al.* (1971).

Finally, the number of mol of ^2H in FA is given by:

$$\text{mol } ^2H \text{ in FA} = \text{mol H in FA} \cdot ^2H \text{ ppm excess in FA} \cdot 10^{-6} \quad \text{(equation 6)}$$

where mol H in FA is the total number of moles of hydrogen in FA and the ^2H_2O enrichment in FA is given in ppm excess.

Application of adjustment factors on data from DLW studies

The derived adjustment factors were applied to data from two previous DLW studies with reindeer in different physiological states to examine the effect of losses of ^2H through sequestration and in faecal solids on the estimates of r_{CO2} and TEE. The first study was conducted on four captive, adult female reindeer (F1, F2, F3 and F4) in summer and in winter (Gotaas *et al.*, 1997) while the second was conducted on three free-living, sub-adult male reindeer (M1, M2 and M3) in winter (Gotaas *et al.*, accepted). The TEE values presented here for reindeer F1, F2, F3 and F4 differ slightly from the previously published values owing to their being re-calculated using slightly altered equations. Moreover, unlike in the original DLW studies (Gotaas *et al.*, 1997; accepted), no adjustment has been made for loss of ^2H through microbially produced methane.

The water equivalence factor derived from the measured loss of ^2H in faecal solids (g $H_2O \cdot g^{-1}$ faeces DM) was combined with an estimate of total faecal DM output to yield an estimate of the apparent water flux due to loss of ^2H in faecal solids. Faecal DM output was estimated by combining an assumed food intake of the pelleted ration (RF-80) in captive female reindeer of 15 g DM · kg^{-1} BM in winter and 30 g DM · kg^{-1} BM in summer (Gotaas & Tyler, unpubl.) and an assumed intake of lichen in free-living male animals of 15 g DM · kg^{-1} BM per day with known digestibilities of RF-80 and lichen

Table 1. Calculation of the loss of exchangeable marker in faeces dry matter in three captive non-pregnant, non-lactating adult female reindeer in summer and in winter.

	Reindeer	Body water enrichment ^2H ppm e[a]	Wash-water amount added g	Faecal DM amount analysed g	Wash-water final enrichment ^2H ppm e[b]	Loss of ^2H in faecal solids, H_2O equivalents g $H_2O \cdot g^{-1}$ faeces DM
Summer	A	87.9	8.760	1.309	2.3	0.17
	B	85.9	11.859	1.799	1.2	0.09
	C	59.9	11.807	1.708	1.4	0.17
Winter	A	138.3	11.802	2.415	5.4	0.19
	B	119.9	11.826	1.907	2.7	0.14
	C	113.1	11.821	1.763	2.6	0.15
Mean (s)		100.8 (28.2)	11.312 (1.251)	1.817 (0.358)	2.6 (1.5)	0.15 (0.04)

[a] Body water enrichment is given as ppm excess relative to the background level prior to injection of isotopes.
[b] Wash-water final enrichment is given as ppm excess relative to 'pure' wash-water (143.69 ppm).

of 78.3% and 86.2%, respectively (Sletten & Hove, 1990).

The water equivalence factor of 0.5618 g $H_2O \cdot g^{-1}$ FA synthesised was applied to the data from the study in summer assuming that the specific net rate of FA synthesis (g FA synthesised · kg^{-1} BM) in the adult females was the same as that recorded in the sequestration study presented here. The net rate of FA synthesis in free-living female reindeer in winter in this part of Norway is < 10 g·d^{-1} (Christiansen *et al.*, 1997) and we therefore assumed that the loss of ^2H through FA synthesis was negligible in DLW studies in both captive and free-living reindeer in winter. Hence, no adjustment for sequestration was made on data from the winter studies.

Isotope analyses

^2H_2O in all samples was converted to hydrogen gas by zinc reduction (Wong *et al.*, 1987) with the modification that 500 mg zinc was used for the reduction. The ^2H content was determined using a SIRA 10 isotope ratio mass spectrometer (VG Isogas, Middlewhich, UK). All analyses were performed in at least three replicates and the mean values were used in subsequent calculations. The coefficient of variation on repeated analysis was less than 0.4%.

Statistical analyses

Water equivalence factors calculated from loss of ^2H attached to faecal solids were compared between groups using the Mann-Whitney U-test. H_0 was rejected at P (two-tailed) ≥ 0.05 in all tests.

The experiments described in this article have been conducted in accordance with current regulations for experimental research involving live animals in Norway.

Results

Exchangeable loss of deuterium in faecal solids

The exchangeable loss of ^2H attached to faecal solids was equivalent to an apparent water flux of 0.09 to 0.17 g $H_2O \cdot g^{-1}$ faeces DM in summer and 0.14 to 0.19 g $H_2O \cdot g^{-1}$ faeces DM in winter (Table 1). However, the difference in the apparent water flux between seasons was not significant ($P > 0.80$) and a mean water equivalence factor of 0.15 g $H_2O \cdot g^{-1}$ faeces DM (s = 0.04, n = 6; Table 1) was therefore used in the subsequent calculations.

Sequestration of deuterium into newly synthesised fat

The mean enrichment of ^2H in the body water of the animals over the 21 day experimental period was 266.9 ppm excess (s = 2.8, n = 4) relative to the control animal. The steady state concentration of ^2H_2O was maintained throughout the experiment by the intake of labelled drinking water as the mean decline in enrichment of ^2H during the experiment was only 0.23 ppm · d^{-1} (s = 0.10, n = 4; Table 2). At the end of the experiment the mean enrichment of ^2H in water produced by sealed bomb combustion of extracted FAs was 41.2 ppm excess (s = 4.1, n = 4) relative to an identically processed sample from the control animal (Table 2). The measured increase in the enrichment of FAs was equivalent to a mean rate of FA synthesis of 75.4 g · d^{-1} (s = 13.2, n = 4; Table 2). Combined with a water equivalence

Table 2. Sequestration of ^2H in fatty acids, rate of fatty acid synthesis and loss of ^2H converted into water equivalents ($g \cdot day^{-1}$) in four young (13 mo.) male reindeer in captivity in summer.

				Carcass Fatty Acids			
Reindeer	Mean BM kg	Mean body water enrichment ^2H ppm e[a]	Mean decline in ^2H ppm·d^{-1}	Total weight in carcass g	Enrichment of combustion water ^2H ppm e[b]	Rate of FA synthesis g·d^{-1}	Total H_2O equivalents g·d^{-1}
E	72.2	262.8	0.28	5234.3	40.9	73.24	41.15
F	64.9	268.0	0.16	6280.0	41.1	86.55	48.63
G	70.7	268.9	0.13	5432.9	46.4	84.18	47.30
H	74.4	267.9	0.35	4723.2	36.4	57.61	32.37
I	76.4			4814.3			
Mean (s)	71.7 (4.4)	266.9 (2.8)	0.23 (0.10)	5297.0 (622.7)	41.2 (4.1)	75.39 (13.20)	42.36 (7.42)

[a] Body water enrichments are given as ppm excess relative to the mean enrichment of the body water of the control animal (reindeer I; 148.94 ppm). By comparison, the mean body water enrichment of all five animals prior to injection of isotope was 147.93 ppm (s = 0.30).

[b] All enrichments of combustion water are given as ppm excess relative to the enrichment of combustion water from FAs from the control animal (reindeer I; 117.60 ppm).

factor of 0.5618 g $H_2O \cdot g^{-1}$ fat synthesised, the sequestration of ^2H corresponded to a mean apparent water flux of 42.36 $g \cdot d^{-1}$ (s = 7.42, n = 4; Table 2).

Error on measurements of J_{H2O} and r_{CO2} and estimates of TEE in DLW studies

Adjustment for loss of ^2H in faecal solids in the DLW study with free-living male reindeer in winter (Gotaas *et al.*, in press) showed that, on average, unadjusted water flux (J_{H2O}) was overestimated by 0.38% (s = 0.05, n = 3, Table 3), r_{CO2} was underestimated by 1.71% (s = 0.36, n = 3, Table 3) while TEE was underestimated by 4.58% (s = 0.35, n = 3, Table 3).

A similar effect was found in captive female reindeer in winter (Gotaas *et al.*, 1997). Failure to adjust for loss of ^2H in faecal solids resulted in an average overestimation of J_{H2O} of 1.30% (s = 0.33, n = 4, Table 3), an underestimation of r_{CO2} of 5.25% (s = 0.46, n = 4, Table 3) and an underestimation of TEE of 4.73% (s = 0.46, n = 4, Table 3). The higher error on TEE in captive reindeer reflects the higher faecal DM output in these animals (Table 3).

Failure to adjust for loss of ^2H by sequestration and attached to faecal solids in captive reindeer in summer resulted in an overestimation of J_{H2O} of 1.42% (s = 0.21, n = 4, Table 3), an underestimation of r_{CO2} of 9.00% (s = 2.34, n = 4, Table 3) and an underestimation of TEE of 9.10% (s = 2.06, n = 4, Table 3). When examining each component separately, we found that adjusting for loss of ^2H through sequestration alone increased the estimated TEE by 3.70% (s = 0.70, n = 4, Table 4) while loss of ^2H attached to faecal solids increased the estimated TEE by 5.87% (s = 1.26, n = 4, Table 4).

Discussion

The potential significance of losses of ^2H in faecal solids and through sequestration has been largely ignored in previous DLW studies in ruminants. For instance, Parker *et al.* (1990) made no mention of adjusting for these non-water losses of ^2H in a study of caribou (*R. t. granti*) and muskoxen (*Ovibos moschatus*) neonates despite the fact that the body mass of the experimental animals increased considerably during the period. Likewise, neither Nagy *et al.* (1990) in a study of two species of marsupials and of black-tailed deer (*Odocoileus hemionus*) nor Nagy & Knight (1994) in a study of springbok antelope (*Antidorcas marsupialis*) nor Fancy *et al.* (1986) in a study of caribou (*R. t. granti*) and reindeer (*R. t. tarandus*) reported making any adjustments to compensate for non-water losses of ^2H. We have shown, however, that unless adjustment is made for losses of ^2H attached to faecal solids and through sequestration in DLW studies with reindeer, TEE can be underestimated by approximately 5% in winter and by approximately 9% in summer.

Exchangeable loss of deuterium in faecal solids

The water equivalence factor due to exchangeable loss of ^2H of 0.15 g $H_2O \cdot g^{-1}$ faeces DM in our rein-

Table 3. Effect of loss of ^2H in faecal solids and through sequestration in three free-living, young (22 mo.) male reindeer (M1, M2 and M3) in winter and in four captive, adult (>4 yrs.), non-pregnant, non-lactating female reindeer (F1, F2, F3 and F4) in summer and in winter.

	Estimated rate of FA synthesis	Estimated rate of faeces production	J_{H2O}			r_{CO2}	TEE		
Reindeer	g · d^{-1}	g DM · d^{-1}	Unadjusted g · d^{-1}	Adjusted g · d^{-1}	Overestimate %	Underestimate %	Unadjusted W · kg^{-1}	Adjusted W · kg^{-1}	Underestimate %
				Free-living male reindeer, Winter					
M1	-	129.9	4504	4485	0.44	2.08	2.260	2.375	4.95
M2	-	133.5	5621	5600	0.36	1.69	2.798	2.932	4.56
M3	-	115.4	5010	4993	0.35	1.36	3.480	3.634	4.24
Mean (*s*)		126.3 (9.6)	5045 (559)	5026 (559)	0.38 (0.05)	1.71 (0.36)	2.845 (0.612)	2.981 (0.631)	4.58 (0.35)
				Captive female reindeer, Winter					
F1	-	270.2	2373	2332	1.73	5.74	1.410	1.488	5.23
F2	-	253.9	3935	3897	0.98	5.50	1.477	1.554	4.99
F3	-	276.7	3785	3743	1.11	4.74	1.726	1.802	4.22
F4	-	257.2	2869	2830	1.36	5.00	1.632	1.708	4.48
Mean (*s*)		264.5 (10.8)	3241 (746)	3200 (746)	1.30 (0.33)	5.25 (0.46)	1.561 (0.144)	1.638 (0.143)	4.73 (0.46)
				Captive female reindeer, Summer					
F1	85.2	514.3	7554	7428	1.67	11.96	2.040	2.306	11.55
F2	95.4	576.1	9726	9584	1.45	9.58	2.616	2.902	9.84
F3	98.1	592.4	12537	12392	1.16	7.96	3.205	3.492	8.22
	86.3	520.8	8989	8862	1.42	6.52	3.977	4.266	6.78
Mean (*s*)	91.2 (6.5)	550.9 (39.2)	9702 (2094)	9567 (2086)	1.42 (0.21)	9.00 (2.34)	2.960 (0.828)	3.242 (0.837)	9.10 (2.06)

deer is 28% higher than the value of 0.117 g H_2O · g^{-1} faeces DM in sheep (Midwood *et al.*, 1993) and 56% higher than the assumed value of 0.096 g H_2O · g^{-1} faeces DM in pigs (Haggarty *et al.*, 1994). Haggarty *et al.* (1994) suggested that the difference in the water equivalence factor between sheep and pigs might be a consequence of the sheep being given a diet rich in cellulose and, hence, producing faeces with a higher proportion of sites available for exchange of ^2H. Our data suggest that the faeces produced by reindeer given a pelleted ration has an even higher proportion of hydrogen exchange sites.

Despite the high water equivalence factor in reindeer compared to both sheep and pigs, the effect of exchangeable loss of ^2H on J_{H2O} was low in free-living reindeer in winter (0.38%) compared to that in sheep (0.75%; Midwood *et al.*, 1993) and in pigs (0.53% in pigs on a restricted diet and 0.98% in pigs fed *ad lib.*; Haggarty *et al.*, 1994). This reflected the very low low estimated faeces DM output in the free-living reindeer in winter (126.3 g · d^{-1}). Faecal DM output in reindeer, however, varies both with season and diet. For instance, the estimated output in captive reindeer in winter was almost identical to that measured in sheep by Midwood *et al.* (1993) (262 g · d^{-1} and 264.5 g · d^{-1}, respectively)

Table 4. Partitioning of non-water losses of ^2H to evaluate the separate effects of loss in faecal solids and through sequestration in studies in four captive, adult (>4 yrs), non-pregnant, non-lactating female reindeer (F1, F2, F3 and F4) in summer.

	Sequestration		Loss in faecal solids		TEE		
Reindeer	Estimated rate of FA synthesis g · d^{-1}	Apparent water flux g · d^{-1}	Estimated production g DM · d^{-1}	Apparent water flux g · d^{-1}	Un-adjusted W · kg^{-1}	Adjusted W · kg^{-1}	Under-estimate %
			Captive female reindeer, Summer, effect of FA synthesis				
F1	85.2	47.9	-	-	2.040	2.133	4.38
F2	95.4	53.6	-	-	2.616	2.729	4.13
F3	98.1	55.1	-	-	3.205	3.319	3.44
F4	86.3	48.5	-	-	3.977	4.093	2.84
Mean (*s*)	91.2 (6.5)	51.3 (3.6)			2.960 (0.828)	3.069 (0.837)	3.70 (0.70)
			Captive female reindeer, Summer, effect of loss of deuterium in faecal solids				
F1	-	-	514.3	78.2	2.040	2.200	7.30
F2	-	-	576.1	87.6	2.616	2.796	6.43
F3	-	-	592.4	90.0	3.205	3.386	5.35
F4	-	-	520.8	79.2	3.977	4.160	4.41
Mean (*s*)			550.9 (39.2)	83.7 (6.0)	2.960 (0.828)	3.136 (0.837)	5.87 (1.26)

but increased to 551.9 g · d^{-1} in summer. By comparison, the faecal DM output in pigs has been found to be 320 g · d^{-1} in animals fed *ad lib.* (Haggarty *et al.*, 1994). The resulting overestimate of J_{H2O} was therefore higher in captive reindeer than in both sheep and pigs (1.30% and 1.42% in captive reindeer in winter and in summer, respectively, but only 0.75% in sheep (Midwood *et al.*, 1993), 0.53% in pigs on a restricted diet and 0.98% in pigs fed *ad lib.* (Haggarty *et al.*, 1994)). Faecal DM output is clearly an important variable determining exchangeable losses of ^2H in highly seasonal animals like reindeer.

Midwood *et al.* (1993) took the further precaution of measuring the non-exchangeable loss of ^2H in faecal solids in sheep. They found the water equivalence factor due to this loss to be 0.013 g $H_2O \cdot g^{-1}$ faeces DM (s = 0.002, n = 4), i.e. approximately 10% of the value derived for the exchangeable loss. Non-exchangeable losses of ^2H were not quantified in the present study. However, assuming that the ratio between exchangeable and non-exchangeable loss of ^2H is similar in sheep and in reindeer, this omission would introduce an error of only approximately 0.5% on the estimated TEE values when applying the adjustment factors on data from DLW studies with both captive and free-living reindeer.

A loss of ^{18}O would reduce the estimated error on r_{H2O}, r_{CO2} and TEE. However, this effect is likely to be small compared to the loss of ^2H due to the dominance of hydrogen over oxygen in most biological materials including faeces (Midwood *et al.*, 1993). Therefore we did not measure the content of ^{18}O in the wash-water from the faecal solids (i.e. exchangeable loss of ^{18}O).

Sequestration of deuterium into newly synthesised fat

The water equivalence factor due to sequestration was 0.5618 g $H_2O \cdot g^{-1}$ FA synthesised in reindeer. The corresponding value in sheep was 0.5536 g $H_2O \cdot g^{-1}$ FA synthesised (calculated from data published by Midwood *et al.* (1993)), in pigs 0.5599 g $H_2O \cdot g^{-1}$ FA synthesised (calculated from data published by Haggarty *et al.* (1991)) and in humans 0.5333 g $H_2O \cdot g^{-1}$ FA synthesised (Haggarty, 1990). The differences between species are small owing to the similarity of the chemical composition of their adipose tissue and, consequently, any species differences in the error introduced to estimates of r_{CO2}, J_{H2O} and TEE by sequestration cannot be due to differences in the stoichiometry of ^2H incorporation but will depend mainly on the rate of FA synthesis. Unlike the water equivalence factor, the rate of FA synthesis differed substantially between species. For instance, the estimated rate of FA synthesis was 75.39 g · d^{-1} in our reindeer compared to 0.42 to 6.37 g · d^{-1} in sheep (Midwood *et al.*, 1993) and 10.2 g · d^{-1} in pigs fed a restricted diet (Haggarty *et al.*, 1994). By comparison, pigs fed *ad lib.* a high

carbohydrate diet had a much higher rate of FA synthesis (119.5 g · d^{-1}; Haggarty *et al.*, 1994).

Sequestration in our reindeer was quantified in the period from late July to mid August, coinciding with the annual peak in the rate of body mass increase and fat deposition recorded in caribou and reindeer (e.g. McEwan, 1968; Ryg & Jacobsen, 1982; Larsen & Blix, 1985; Tyler, 1987). Furthermore, the animals investigated (young males) are known to have proportionally the largest body mass increase during this period (McEwan, 1968; Ryg & Jacobsen, 1982). Given the age and sex of the animals, the timing of the study and the fact that they were given a high-quality ration, we assume that the estimated net rate of FA synthesis (75.39 g·d^{-1}) and hence, the rate of incorporation of ^2H, was close to maximal. Our value, in fact, compares closely with the net rate of FA synthesis (approximately 70 g · d^{-1}) recorded in free-living adult female caribou (*R. t. groenlandicus*) on a natural pasture in summer (Adamczewski *et al.*, 1987).

Error on measurements of r_{CO2} and estimates of TEE in DLW studies

The ratio of [errors on r_{CO2}]:[errors on estimates of TEE] varied substantially between experiments. The non-water losses of ^2H generated an underestimate of r_{CO2} of 1.71% in free-living males in winter, 5.25% in captive females in winter and 9.00% in captive females in summer, respectively. These errors, in turn, translated into underestimates of TEE of 4.58%, 4.73% and 9.10%, respectively, in these three experiments. The ratios of [errors on r_{CO2}]:[errors on estimates of TEE] in the different experiments were, thus, 0.37, 1.11 and 0.99, respectively. These ratios deviate both from unity and from each other because the estimate of r_{CO2} depends on the proportion of the water loss exposed to fractionation (X), while the estimate of TEE depends both on X and the respiratory quotient (RQ) and the values of both X and RQ vary from experiment to experiment (Gotaas *et al.*, 1997; in press).

Conclusions and perspectives

Loss of ^2H attached to faecal solids and through sequestration, so-called non-water losses, can be responsible for significant underestimation of TEE in reindeer determined using the DLW method. Given the net rate of lipid synthesis and the rate of faecal DM output described in the present study, the non-water losses of ^2H caused TEE to be underestimated by approximately 5% in winter and approximately 9% in summer. The estimates of TEE can be significantly improved by the application of the adjustment factors described in this paper. Knowledge of the net rates of lipid synthesis and of faecal DM output is a prerequisite for making the necessary adjustments.

Energy is the universal currency used to measure and assess production and the efficiency of production in animals. At present, the DLW method is the only practical way of estimating TEE in free-living individuals over significant periods (days or weeks in large animals). Successful application of the DLW method, however, requires knowledge of the potential sources of error inherent in it and the generation of reliable adjustment factors to correct for these. The water equivalence factor used in the correction for non-water losses of ^2H in FA in the present study (i.e. g H_2O · g^{-1} FA) can be assumed to be constant in reindeer because the composition of FA is unlikely to vary greatly throughout the year. This assumption may not be valid for faeces, however, and the water equivalence factor (g H_2O · g^{-1} faeces DM) might have to be revised depending on season.

Net rates of FA synthesis and voluntary food intake, and presumably also faecal DM output, are seasonally highly variable in reindeer and the latter, in particular, is poorly documented. Better data on faecal DM output combined with the water equivalence factors described in this study and known net rates of FA synthesis would enable complete correction for two important non-water losses of ^2H in any ecological or production context in which the energetics of free-living reindeer are studied.

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References

Adamczewski, J. Z., Gates, C. C., Hudson, R. J. & Price, M. A. 1987. Seasonal changes in body composition of mature female caribou and calves (*Rangifer tarandus groenlandicus*) on an arctic island with limited winter resources. – *Canadian Journal of Zoology* 65: 1149–1157.

Bøe, U.-L. & Jacobsen, E. 1981. Fôringsforsøk med ulike typer fôr til rein. (Feeding trial with different types of feed for reindeer). – *Rangifer* 1: 39–43. (In Norwegian).

Christiansen, H. R., Tyler, N. J. C. & Johansen, O. 1997. Bruker simler i Finnmark kroppsreserver om vinteren? (Do female reindeer in Finnmark deplete their body reserves in winter?) – *Reindriftsnytt* 31 (3): 15–19 (In Norwegian).

Fancy, S. G., Blanchard, J. M., Holleman, D. F., Kokjer, K. J. & White, R. G. 1986. Validation of doubly labeled water method using a ruminant. – *American Journal of Physiology* 251: R143–R149.

Garton, G. A. & Duncan, W. R. H. 1971. Fatty Acid Composition and Intramolecular Structure of Triglycerides from Adipose Tissue of the Red Deer and the Reindeer. – *Journal of the Science of Food and Agriculture* 22: 29–33.

Garton, G. A., Duncan, W. R. & McEwan, E. H. 1971. Composition of adipose tissue triglycerides of the elk (*Cervus canadensis*), caribou (*Rangifer tarandus groenlandicus*), moose (*Alces alces*), and white-tailed deer (*Odocoileus virginianus*). – *Canadian Journal of Zoology* 49: 1159–1162.

Gotaas, G. & Tyler, N. J. C. 1995. Faeces is a reliable source of body water for measuring tritium in reindeer in summer and in winter. – *Rangifer* 15: 63–70.

Gotaas, G., Milne, E., Haggarty, P. & Tyler, N. J. C. 1997. Use of feces to estimate isotopic abundance in doubly labeled water studies in reindeer in summer and winter. – *American Journal of Physiology* 273: R1451–R1456.

Gotaas, G., Milne, E., Haggarty, P. & Tyler, N. J. C. 2000. Energy expenditure of free-living reindeer estimated by the doubly labelled water method. – *Rangifer,* (in press).

Haggarty, P. 1990. The effect of isotope sequestration and exchange. – *In:* A. M. Prentice, ed. *The doubly-labelled water method for measuring energy expenditure - Technical recommendations for use in humans,* pp. 114-146. Vienna: International Atomic Energy Agency.

Haggarty, P., Wahle, K. W., Reeds, P. J. & Fletcher, J. M. 1987. Whole body fatty acid synthesis and fatty acid intake in young rats of the Zucker strain (Fa/- and fa/fa). – *International Journal of Obesity* 11: 41–50.

Haggarty, P., McGaw, B. A., Fuller, M. F., Christie, S. L. & Wong, W. W. 1991. Water hydrogen incorporation into body fat in pigs: effect on double/triple-labeled water method. – *American Journal of Physiology* 260: R627–R634.

Haggarty, P., Franklin, M. F., Fuller, M. F., McGaw, B. A., Milne, E., Duncan, G., Christie, S. L. & Smith, J. S. 1994. Validation of the doubly labeled water method in growing pigs. – *American Journal of Physiology* 267: R1574–R1588.

Haggarty, P., Robinson, J. J., Ashton, J., Milne, E., Adam, C. L., Kyle, C. E., Christie, S. L. & Midwood, A. J. 1998. Estimation of energy expenditure in free-living red deer (*Cervus elaphus*) with the doubly labelled water method. – *British Journal of Nutrition* 80: 263–372.

Jungas, R. L. 1968. Fatty acid synthesis in adipose tissue incubated in tritiated water. – *Biochemistry* 7: 3708–3717.

Larsen, T. S. & Blix, A. S. 1985. Seasonal changes in total body water, body composition and water turnover in reindeer. – *Rangifer* 5 (1): 2–9.

Lifson, N., Gordon, G. B. & McClintock, R. 1955. Measurement of total carbon dioxide production by means of $D_2{}^{18}O$. – *Journal of Applied Physiology* 7: 704–710.

McEwan, E. H. 1968. Growth and development of the barren-ground caribou. II. Postnatal growth rates. – *Canadian Journal of Zoology* 46: 1023–1029.

McGaw, B. A., Milne, E. & Duncan, G. J. 1988. A rapid method for the preparation of combustion samples for stable carbon isotope analysis by isotope ratio mass spectrometry. – *Biomedical and Environmental Mass Spectrometry* 16: 269–273.

Midwood, A. J. 1990. *Application of the doubly labelled water technique for measuring CO_2 production to sheep.* Ph.D. thesis, University of Aberdeen.

Midwood, A. J., Haggarty, P., McGaw, B. A. & Robinson, J. J. 1989. Methane production in ruminants: its effect on the doubly labeled water method. – *American Journal of Physiology* 257: R1488–R1495.

Midwood, A. J., Haggarty, P. & McGaw, B. A. 1993. The doubly labeled water method: errors due to deuterium exchange and sequestration in ruminants. – *American Journal of Physiology* 264: R561–R567.

Midwood, A. J., Haggarty, P., McGaw, B. A., Mollison, G. S., Milne, E. & Duncan, G. J. 1994. Validation in sheep of the doubly labeled water method for estimating CO_2 production. – *American Journal of Physiology* 266: R169–R179.

Nagy, K. A. 1987. Field metabolic rate and food requirement scaling in mammals and birds. – *Ecological Monographs* 57: 111–128.

Nagy, K. A. 1994. Field bioenergetics of mammals: What determines field metabolic rates? – *Australian Journal of Zoology* 42: 43–53.

Nagy, K. A. & Knight, M. H. 1994. Energy, water, and food use by springbok antelope (*Antidorcas marsupialis*) in the Kalahari Desert. – *Journal of Mammalogy* 75: 860–872.

Nagy, K. A., Sanson, G. D. & Jacobsen, N. K. 1990. Comparative field energetics of two macropod marsupials and a ruminant. – *Australian Wildlife Research* 17: 591-599.

Parker, K. L., White, R. G., Gillingham, M. P. & Holleman, D. F. 1990. Comparison of energy metabolism in relation to daily activity and milk consumption by caribou and muskox neonates. – *Canadian Journal of Zoology* 68: 106–114.

Ryg, M. & Jacobsen, E. 1982. Seasonal changes in growth rate, feed intake, growth hormone, and thyroid hormones in young male reindeer (*Rangifer tarandus tarandus*). – *Canadian Journal of Zoology* 60: 15–23.

Sletten, H. & Hove, K. 1990. Digestive studies with a feed developed for realimentation of starving reindeer. – *Rangifer* 10: 31–37.

Tyler, N. J. C. 1987. Body composition and energy balance of pregnant and non-pregnant Svalbard reindeer during winter. – *Symposia. Zoological Society of London* 57: 203–229.

Wong, W. W., Lee, L. S. & Klein, P. D. 1987. Deuterium and oxygen-^{18} measurements on microliter samples of urine, plasma, saliva, and human milk. – *American Journal of Clinical Nutrition* 45: 905–913.

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