University of Groningen

Validity of the doubly labeled water method for estimating CO2 production in mice under different nutritional conditions

Guidotti, Stefano; Meijer, Harro; van Dijk, Gertjan

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Link to publication in University of Groningen/UMCG research database

Validity of the doubly labeled water method for estimating CO_2 production in mice under different nutritional conditions

Stefano Guidotti,[1,2] Harro A. J. Meijer,[1] and Gertjan van Dijk[1,2]

[1]*Center for Isotope Research, Energy and Sustainability Research Institute Groningen, University of Groningen, Groningen, The Netherlands;* [2]*Center for Behavior and Neurosciences, Unit Neuroendocrinology, University of Groningen, Groningen, The Netherlands*

Guidotti S, Meijer HA, van Dijk G. Validity of the doubly labeled water method for estimating CO_2 production in mice under different nutritional conditions. *Am J Physiol Endocrinol Metab* 305: E317–E324, 2013. First published May 28, 2013; doi:10.1152/ajpendo.00192.2013.— The doubly labeled water (DLW) technique is used to assess metabolic rate (MR) in free-living conditions. We investigated whether differences in the nutritional and body adiposity status affect validity of the assessment of CO_2 production (rCO_2) by the DLW technique. To serve this purpose, we compared calculated rCO_2 by the DLW method to actual CO_2 production concomitantly measured in an indirect calorimetry setup over a 3-day period in mice fed with a low-fat (LF) diet or an obesogenic high-fat/high-sucrose (HF) diet. To uncover a potential effect of body composition on DLW accuracy, the HF-fed group was further subdivided in a diet-induced obesity-prone (DIO) and diet-induced obesity-resistant (DR) group. Furthermore, we assessed the influence of different sampling protocols, duration, and methodology of calculation. An excellent match was found between rCO_2 assessed by the two methods in the LF-fed mice (least discrepancy $-0.5 \pm 1.1\%$). In contrast, there was a consistent overestimation of rCO_2 by the DLW technique in the HF-fed animals compared with actual CO_2 production independent from body mass gain (least discrepancy DR $+15.9 \pm 2.2\%$, DIO $+18.5 \pm 3.2\%$). The least discrepancies were found when two-pool model equations and the intercept method were used to calculate the body water pool. Furthermore, the HF group presented different equilibration kinetics of ^2H and ^{18}O and a lower dilution space ratio between the two. We recommend particular caution when using the DLW method for MR assessment in HF-fed animals and potentially humans because of the overestimation of rCO_2.

body composition; diet-induced obesity; doubly labeled water; metabolic rate; validation

THE CURRENT EPIDEMIC OF OBESITY has spurred the need for understanding how energy balance and body weight are regulated and how derangements in these can develop. The doubly labeled water (DLW) technique has served this demand because it is an isotopic tracer method ideally suited for measuring metabolic rate (MR) and body composition in free-living conditions. As such, the DLW method has been used in laboratory as well as field studies to assess MR in a variety of species, including humans, migratory birds, and rodents (19, 27, 39).

DLW is a water mixture in which the natural abundant forms of hydrogen (^1H) and oxygen (^{16}O) have been partially or totally replaced with the low abundant stable isotopic forms (^2H and ^{18}O, respectively). The method is based on the straightforward principle that hydrogen leaves the body primarily via water turnover, whereas oxygen leaves the body both via water turnover and via CO_2 production. The difference between the two elimination rates allows the estimation of the rate of CO_2 production (rCO_2) over time. By introducing a known mass of the DLW mixture in the body by injection or drinking, enrichments of body fluids by ^2H and ^{18}O are determined at an initial and final time point from which elimination rates can be determined. rCO_2 can be converted to MR by using an estimated or measured respiratory quotient (RQ) on the basis of the equation of Weir (38). This allows assessment of MR and body composition in conditions where confinement in an indirect calorimetry setup (i.e., where the actual rates of rCO_2 and O_2 consumption, rO_2, are assessed) is impossible to perform, since it would conflict with the study design. Examples of these are assessment of MR in animals living in a social context (3), in flight energy expenditure during bird migration (39), in lactation energetics (Guidotti S, Jónás I, Schubert K, Garland T Jr, Meijer H, Scheurink A, and van Dijk G, unpublished observations), or during sleep deprivation (1, 2).

Despite the fact that the principle of DLW technique for calculation of MR is rather straightforward, it is based on several assumptions, as reported originally by Lifson and colleagues (20, 21), that may not always be met in experimental settings. Violation of these assumptions can have a negative impact on the validity of the assessment of rCO_2 and hence, MR (24, 31). One of the assumptions, particularly relevant in light of the use of the DLW technique for studying derangements in energy balance and obesity, is that hydrogen and oxygen do not compartmentalize beyond the water and CO_2 pool. However, hydrogen and oxygen probably violate this assumption since they could be incorporated into nonaqueous molecules via ionic exchange or anabolic metabolism, resulting in larger pool sizes and isotopic fractional washout rates. In fact, ^2H incorporation into C-H bonds has been used in biomedical sciences to measure in vivo lipogenesis (36) or synthesis of macromolecules such as DNA (12).

To our knowledge, studies in mammals that compared rCO_2 assessed by indirect calorimetry to the level obtained by the DLW technique generally find overestimation by the latter (20, 23), which might be due to nutritional and/or energy balance parameters. Therefore, the main aim of this study was to investigate whether differences in the nutritional status and energy balance affect the validity of rCO_2 assessed by the DLW technique. To serve this purpose, we compared rCO_2 calculated by the DLW method with actual CO_2 production concomitantly measured using a calibrated CO_2 sensor in an indirect calorimetry setup over a 3-day period in mice fed a

Address for reprint requests and other correspondence: G. van Dijk, Univ. of Groningen, Nijenborgh 7, 9747AG Groningen, The Netherlands (e-mail: gertjan.van.dijk@rug.nl).

low-fat (LF) diet and an obesogenic high-fat/high-sucrose (HF) diet. To uncover a potential effect of body composition on DLW validity, the HF-fed group was further subdivided in a diet-induced obesity-prone (DIO) and diet-induced obesity-resistant (DR) group. Furthermore, we assessed the influence of different sampling protocols, duration, and method of calculation.

MATERIALS AND METHODS

Animals and housing. Thirty-two male outbred HSD:icr mice of 14 wk were initially used for this study. The mice were housed individually on a 12:12-h light-dark cycle (lights on 9 AM, lights off 9 PM) under temperature-controlled conditions (21 ± 1°C), with food and water ad libitum, in plexiglas cages (Macrolon Type II; UNO Roestvaststaal, Zevenaar, The Netherlands) with Envirodr wood shavings and a gnawing stick. After initial inclusion, eight of the mice were maintained on regular control (LF) laboratory chow (energy content: 15.9 kJ/g; 13% fat, 63% starch, and 24% protein, RMH-B 2181; HopeFarms, Woerden, The Netherlands), whereas the other 24 were switched to a 45% HF diet (energy content: 19.7 kJ/g; 45% fat, 18% starch, 13% sucrose, and 24% protein) 6 wk prior the experiment. On the basis of body mass gain over the course of HF feeding, mice were assigned to a DR or DIO group.

Experimental design. Under nonrestrictive conditions, LF- and HF-fed mice received an intraperitoneal injection of DLW (~0.15 g weighed to the nearest 0.0001 g of enriched water consisting of 33.3% ^{2}H and 66.6% ^{18}O). Exactly 1, 2, and 3 h after dosing of the DLW mixture, blood was sampled from the mice by tail snip and flame-sealed into glass capillaries until determination of isotopic enrichment. Directly after sampling of the last initial sample, mice were transferred to indirect calorimetry cages with housing conditions similar to their home cage for 3 consecutive days. To reduce stress of novelty, wood shaving from their home cage was transferred as well. Exactly 24, 48, and 72 h after the third initial, blood was again sampled by tail snip and again flame-sealed into glass capillaries. For the 24- and 48-h blood sample, indirect calorimetry was interrupted shortly for ~10 min. After the 72-h blood sample, HF-fed mice were euthanized for carcass analysis. These and all other experimental procedures were approved and guided by the local Animal Experimentation Committee of the University of Groningen.

Indirect calorimetry. The open-circuit indirect calorimeter system allowed determination of rO$_2$ (l/h) and rCO$_2$ (l/h) (37). In brief, O$_2$ and CO$_2$ concentrations of dried inlet and outlet air from each chamber were measured with paramagnetic O$_2$ analyzer (Sevomex Xentra 4100, Crowborough, UK) and CO$_2$ by an infrared gas analyzer (Servomex 1440). The system recorded the differential in O$_2$ and CO$_2$ between dried reference air and dried air from metabolic chambers. O$_2$ and CO$_2$ analyzers were calibrated with two gas mixtures with known concentrations of O$_2$ and CO$_2$ prior to each measurement. Flow rate of inlet air was measured with a mass flow controller (Type 5850; Brooks, Rijswijk, The Netherlands) and set at 20 l/h. Of the respiration air, a subsample was passed at a rate of 6 l/h through the drying system (3 Å molecular sieve drying beads; Merck, Darmstadt, Germany) and subsequently through the gas analyzers. Data were collected every 10 min for each animal and automatically stored on a computer. The RQ was defined as rCO$_2$ divided by rO$_2$. MR was calculated according to Weir (38):

$$\text{MR (kJ)} = rCO_2\left(\frac{15.457}{RQ} + 5.573\right)$$

Body composition. Body composition was determined by both the isotope dilution method (plateau/intercept) and direct carcass analysis. Carcasses were dried till constant weight at 103°C [ISO 6496–1983(E)], which was usually the case after 4 h, and this was followed

by fat extraction with petroleum ether (Boom, Meppel, The Netherlands) in a soxhlet apparatus.

Analysis of the DLW samples. Determinations of ^{2}H/^{1}H and ^{18}O/^{16}O ratios in blood samples were performed at the University of Groningen Center for Isotope Research. A detailed description of the analytical procedures followed in our laboratory is described elsewhere (9). Shortly, samples were prepared by microdistillation (cryodistillation) in a vacuum line, first heating the broken capillaries and then cryogenically trapping the emerging water vapor with liquid nitrogen. Water samples were stored in glass vials and then automatically injected into a Hekatech high-temperature pyrolysis (Hekatech, Wegberg, Germany) unit (8), in which the injected water reacted with glassy carbon at a temperature of 1,420°C. The resultant H$_2$ and CO gasses, emerging into a continuous helium flow through the system, were then led through a gas chromatography column to separate the two gases in time and finally fed into an isotope ratio mass spectrometer (GV Instruments IsoPrime, Manchester, UK) for the analysis of δ^{18}O and δ^2H. Measurements were corrected for memory effects using an algorithm described previously (9).

For each analytical series, at least three internal water standards were chosen to cover the entire enrichment range of the samples. Those standards samples were prepared and analyzed, with the same methods followed (distilled and undistilled).

Samples were measured in duplicates, unless a flaw was detected in the flame-sealing step. Maximum allowed duplicate differences were 5 and 2% of the value for δ^2H values and 0.8 and 1.5% of the value for δ^{18}O values (9). If the differences exceeded the above-mentioned levels, we examined the data critically and omitted the aberrant value, performing further calculations on a single replicate, and/or analyzed a third sample if available. More detailed information on how the analytical error propagating into rCO$_2$ was estimated is described elsewhere (9).

Isotope dilution spaces. Estimation of the body water pools for ^{2}H (N$_D$) and ^{18}O dilution space (N$_O$), using the plateau method, was performed according to the following equation:

$$\text{N (mol)} = \text{mol}_{inj}\frac{(E_{ini} - E_{inj})}{(E_{bkg} - E_{ini})}$$

where N represents the dilution space or body water pool for ^{2}H or ^{18}O expressed in moles. E$_{ini}$ and E$_{bkg}$ are the enrichments at equilibrium and at background in the body, respectively. E$_{inj}$ is the enrichment of the injected water. E is expressed in parts per million.

Estimation of the body water pools for ^{2}H (N$_D$) and ^{18}O (N$_O$) by the intercept method was performed by extrapolating the E$_{ini}$ back to the time of injection according to the following equation, with modifications (18):

$$E_{ini-ic} = \left(E_{ini} - E_{bkg}\right)e^{k_i(t_{ini}-t_{inj})} + E_{bkg}$$

where E$_{ini-ic}$ is now the initial value to be used for the body water pools calculation, t$_{ini}$ − t$_{inj}$ is the time between injection and the initial sampling (in h), and k$_i$ is the ^{2}H or ^{18}O turnover rate in h^{-1}.

Isotope turnover rates were calculated as follows:

$$k \text{ (mol/h)} = [\log_e(E_{ini} - E_{bkg}) - \log_e(E_{fin} - E_{bkg})]/t$$

where E$_{fin}$ represents the enrichment level at final sample and *t* the time (in h) between final and initial sample. The initial body water pool TBW$_i$ was calculated by the isotope dilution method. TBW$_i$ as percentage of initial body mass was applied to the final body mass to estimate the final pool sizes.

For the multisample methodology the k values were calculated from the curves fitted along the values of samples collected during the 3-day measurement by using the 2-h time point as initial. Enrichment values above background were log transformed and curve-fitted; k values were derived from the slope of the regression line (least-squares fit).

Calculation of rCO₂. Several equations, presented in Supplemental Table S1 (supplemental data for this article can be found on the *AJP-Endocrinology and Metabolism* website),were employed for the calculation of rCO₂. N used in the equations was an average between initial and final body water pool. The first equation, applied in 1966 by Lifson and McClintock (21), used only the ^{18}O dilution space ("one-pool model") and considered only equilibrium fractionation factors at 24°C (see Supplemental Table S1, *Eq. 1*). Speakman further updated this equation by using a combination of in vivo kinetics and equilibrium fractionation factors at 37°C and assuming that they contribute in a ratio of 3:1 to the fractionated losses (see Supplemental Table S1, *Eq. 2*) (31). In two-pool model equations, both observed isotope pool sizes, N_D and N_O, are used in the estimation of rCO₂. The first equation used individual isotope spaces and the fractionation factors in Supplemental Table S1, *Eq. 2* (see Supplemental Table S1, *Eq. 3*) (4). In 1986 and then in 1988, Schoeller and colleagues (27–29) updated the latter equation by using individual dilution space with fixed average pool size for oxygen and hydrogen pools relative to the body water pool (i.e., N_O 1.01 and N_D 1.04 times greater than actual dilution space and with a dilution space ratio of 1.03). The fractionation factors were estimated by equilibrium processes at 37°C, with fractionated water loss being equivalent to 2.3 times rCO₂ (see Supplemental Table S1, *Eq. 4*).

Racette et al. (26) further updated Schoeller's equation in 1994 proposing that the population pool ratio was 1.034 (Supplemental Table S1, *Eq. 8*). Speakman et al. (32) also proposed a two-pool equation in 1993, but using a group dilution ratio, and they adopted the same fractionation factors as the ones used in Supplemental Table S1, *Eq. 4*. The equation was later update by Speakman (31) in 1997, using still a group dilution ratio but with the fractionation factors used in Supplemental Table S1, *Eq. 2* (see Supplemental Table S1, *Eq. 6*) (31).

Finally, in 1980, Nagy (24) proposed an equation that does not use turnover rates (i.e., K_D and K_O) but does use the log ratios of the initial and final isotope concentrations and directly incorporates initial and final pool sizes (N) as well as initial and final body mass of the animal under study (Supplemental Table S1, *Eq. 7*).

Apart from Supplemental Table S1, *Eqs. 1* and *7*, all equations are in fact only minor variations of each other. Yet, especially the choices for the level of fractionated water loss have a significant influence on the result. However, quantifying this fractionation experimentally is cumbersome (11, 14).

Statistical analysis. The data are expressed as means ± SE. Analysis was performed in Statistica (Statsoft). Multiple hour (initials) and day (finals) differences were tested with repeated-measures ANOVA (RM-ANOVA), with time as within factor and group as between factor. To test differences between groups, a general linear model ANOVA was used, followed by Tukey's honestly significant difference post hoc testing.

RESULTS

Body mass. After being on the HF diet for 6 wk, eight of the mice that had the lowest weight gain were assigned to the DR group (n = 8; Δmass −0.05 ± 0.50 g), whereas another eight with the highest weight gain were assigned to the DIO group (n = 8; Δmass 6.12 ± 0.60 g). Using body masses on the day just before indirect calorimetry was started (*day 0*; Fig. 1, *top left*), ANOVA revealed group effects [$F_{(2,21)}$ = 13.69, $P <$ 0.001], and post hoc analysis showed that mice in the HF-DIO group weighed significantly more than those in the HF-DR and LF groups. No differences were found between the HF-DR group and the LF group. In retrospect, the HF-DIO mice also weighed more than the HF-DR mice at the start of HF feeding [$F_{(1,14)}$ = 16.61, $P <$ 0.01]. Percent body fat (see Fig. 1, *top right*) determined by isotopic dilution (see below) was affected by group [$F_{(2,21)}$ = 12.63, $P <$ 0.001], and post hoc analysis showed that the HF-DIO mice had more fat relative to the LF mice (150 ± 8%) and HF-DR mice (113 ± 7%). No significant differences in percent body fat were found between HF-DR mice and LF mice (17 ± 16%).

Over the course of metabolic assessment by DLW/indirect calorimetry, HF-DIO mice reduced body mass slightly, whereas other groups remained stable [$F_{(2,21)}$ = 5.52, $P <$ 0.05; see Fig. 1, *top left*].

Indirect calorimetry. During the placement of mice in the indirect calorimetry cages, the actual rCO₂, rO₂, and RQ were assessed over 3 consecutive days. A summary of the indirect calorimetry data and the calculated MR is presented in Table 1.

There was a different time course between groups [$F_{(4,42)}$ = 8.59, $P <$ 0.001], rO₂ [$F_{(4,42)}$ = 4.45, $P <$ 0.01], and RQ
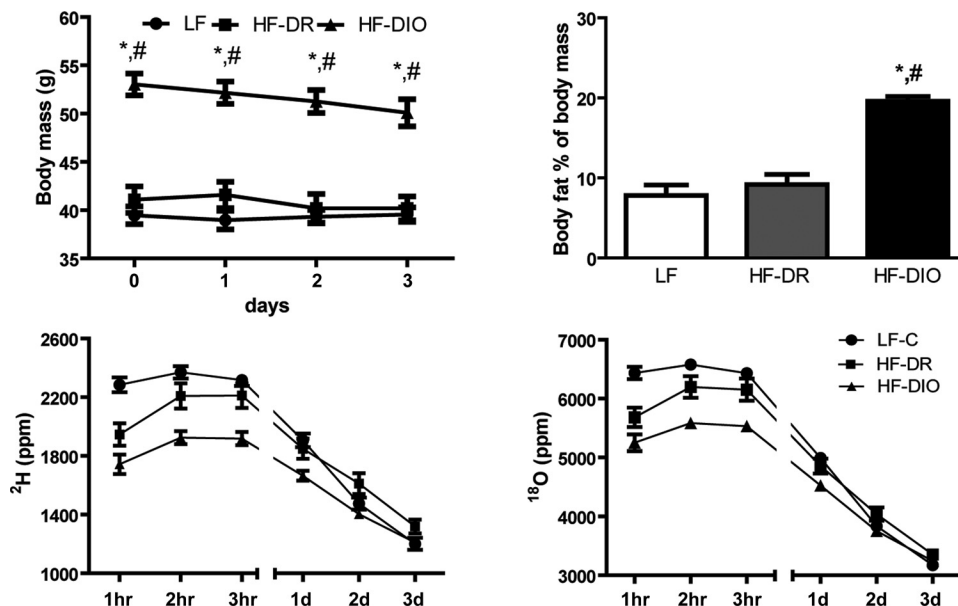
Fig. 1. Body mass change over the course of the measurement (*top left*) and %body fat calculated by the isotope dilution method using the 2-h initial plateau method (*top right*) in high-fat/high-sucrose (HF)-fed diet-induced obesity-prone (DIO; ▲ or black bar) and diet-induced obesity-resistant mice (DR; ■ or gray bar) and in LF-fed (● or open bar) mice. Isotopic equilibration and elimination curves in these groups for ^{2}H and ^{18}O are shown at *bottom left* and *bottom right*, respectively. Data are expressed as means ± SE. Tukey post hoc *LF vs. HF-DR or HF-DIO; #HF-DR vs HF-DIO.

Table 1. *Indirect calorimetry measurements during the 3-day validation study*

	LF	HF-DR	HF-DIO
rCO₂, ml/h			
Day 1	112.6 ± 3.8	100.3 ± 6.1	108.4 ± 2.2
Day 2	116.6 ± 3.1	95.7 ± 5.2	105.0 ± 1.4
Day 3	118.9 ± 2.9	96.1 ± 5.0	104.8 ± 1.8
rO₂, ml/h			
Day 1	112.9 ± 4.9	109.3 ± 7.1	121.6 ± 3.2
Day 2	116.6 ± 4.4	104.4 ± 6.2	118.3 ± 2.3
Day 3	117.3 ± 3.5	104.3 ± 5.9	117.9 ± 2.5
RQ, CO₂/O₂			
Day 1	1.00 ± 0.01	0.92 ± 0.01	0.89 ± 0.01
Day 2	1.00 ± 0.01	0.92 ± 0.01	0.89 ± 0.01
Day 3	1.02 ± 0.01	0.92 ± 0.01	0.89 ± 0.01
MR, kJ/day			
Day 1	56.9 ± 2.3	54.0 ± 3.4	59.6 ± 1.5
Day 2	58.8 ± 2.0	51.5 ± 3.0	57.9 ± 1.0
Day 3	59.4 ± 1.7	51.6 ± 2.9	57.8 ± 1.2

Data are expressed as means ± SE. LF, low-fat diet; HF, high-fat/high-sucrose diet; DR, diet-induced obesity-resistant group; DIO, diet-induced obesity-prone group; rCO₂, rate of CO₂ production; rO₂, rate of oxygen production; RQ, respiratory quotient; MR, metabolic rate.

$[F_{(4,42)} = 10.49\ P < 0.001]$, with levels of rO_2 and rCO_2 increasing in the LF group, whereas they were reduced in the HF-fed groups. A similar time × group interaction effect was observed on MR $[F_{(4,42)} = 5.60, P < 0.01]$, with MR going up in the LF group and down in the HF-fed groups. Between-group effects on MR did not reach statistical significance. When body mass was added as covariate, we observed a similar time × group interaction $[F_{(4,40)} = 4.27, P < 0.01]$. Between-subject analysis revealed a lower MR in the HF-DR group compared with the HF-DIO and LF groups $[F_{(2,20)} = 5.98, P < 0.01]$. The RQ was affected by group $[F_{(2,21)} = 42.03, P < 0.001]$, with both HF-fed groups having a lower RQ than the LF-fed group.

Isotope equilibration and body composition. Blood isotopic enrichments (E_{ini}) of ^2H and ^{18}O at 1, 2, and 3 h after injection of the DLW mixture are presented in Fig. 1, *bottom left* and *bottom right*, respectively. RM-ANOVA revealed a significant time × group interaction effect on E_{ini} ^2H and ^{18}O [for ^2H, $F_{(4,42)} = 5.45\ P < 0.01$; for ^{18}O, $F_{(4,40)} = 3.50\ P < 0.05$], with those in the HF-DIO mice being lowest and those in the LF-fed mice highest. Furthermore, E_{ini} was generally highest at the 2- or 3-h time point, with the 1st hour clearly being lower than the 2nd and 3rd hours for both ^{18}O and ^2H. The E_{ini} $^{18}O/^2H$ ratio showed a significant time × group interaction $[F_{(4,40)} = 4, 90, P < 0.01$; data not shown], with that of the HF-fed groups decreasing significantly over time. The LF group showed only a modest decrease in the E_{ini} $^{18}O/^2H$ ratio over the 2nd and 3rd hours (data not shown).

Initial isotope dilution spaces and body composition. Dilution spaces (N) were calculated at 1, 2, and 3 h after DLW injection by applying the plateau method as well as the intercept method for both ^2H and ^{18}O. Only those calculated by the intercept method using a fixed time point for the final at 24 h are shown in Fig. 2, *top left*. Those calculated by the plateau method yielded similar interactions and differences.

HF-DIO mice had the highest N_O (N_D showed similar differences) at the different time points and the LF-fed mice the lowest N_O. When 1-h initials of ^{18}O (^2H yielded similar results) were used for the determination of N_O by the plateau method, they yielded an ~12% higher value than values calculated from the 2nd- or 3rd-hour initial in the HF-fed groups (see Fig. 2, *top left*). In the LF group, the difference between 1 and 2- to 3-h initial N_O was only ~2% [time × group $F_{(4,40)} = 4.25$, $P < 0.01$].

Dilution space ratios (N_D/N_O) ranged from 0.99 to 1.04 for the plateau method and from 1.02 to 1.07 for the intercept method. RM-ANOVA on N_D/N_O ratios obtained by the plateau method (see Fig. 2, *top right*) revealed a significant effect of time $[F_{2,40} = 72, P < 0.001]$ and group $[F_{(2,20)} = 5, P < 0.05]$. Post hoc analysis revealed that HF-fed mice had significantly lower N_D/N_O than LF-fed mice. There was no effect of time on N_D/N_O calculated by the intercept approach, but group differences for N_D/N_O persisted $[F_{(2,20)} = 10.9, P < 0.001]$. Overall N_D/N_O calculated by the intercept method was 4–5% higher than that calculated by the plateau method.

Fig. 2. ^{18}O dilution space (N_O; *top left*) and ^2H (N_D)/N_O dilution space ratio (*top right*) calculated by the plateau method in HF-fed DIO (black bar) and DR (gray bar) mice and in LF-fed (open bar) mice. In the HF-DR and HF-DIO groups, %deviation of body water measured by carcass desiccation and body water determined by isotope dilution method by plateau (*bottom left*) and by intercept (*bottom right*) for N_O is shown (the values based on isotope dilution are higher). Data are expressed as means ± SE. *LF vs. HF-DR or HF-DIO; #HF-DR vs HF-DIO.
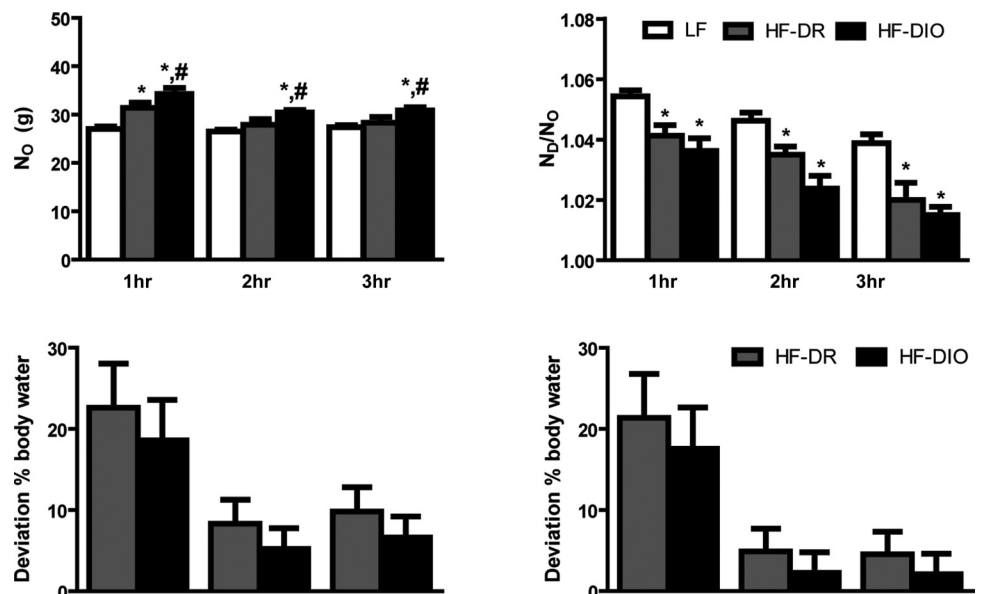
The body water pool assessed by carcass analysis in HF-DIO and HF-DR mice (following desiccation and soxhlet fat extraction), expressed as percentual deviation from the body water pool estimated by ^{18}O dilution, is shown in Fig. 2. Deviations are shown with initials taken at 1, 2, and 3 h and using the "plateau" (Fig. 2, *bottom left*) and "intercept" method, using the final on *day 3* (Fig. 2, *bottom right*). E_{ini} ^{18}O at 1 h resulted in an ~20% overestimation of body water pool relative to the level obtained by the desiccation method. This difference was smaller at 2 and 3 h, particularly when the intercept method was used. None of these levels were different between HF-DIO and HF-DR mice.

Isotope turnovers. Turnover rates of ^{18}O (k_O) and ^2H k_D calculated from logarithmic decline using initials and finals were used for calculation of rCO_2 (from $k_O - k_D$) and for assessment of the body water flux linked to oxygen turnover (by k_D/k_O) in the different experimental groups, and these are shown in Fig. 3.

First, the relevance of the initial time points (i.e., at 1, 2, and 3 h) for $k_O - k_D$ and k_D/k_O in the different diet groups was investigated by using the enrichment levels of ^{18}O and ^2H at the same final point (we chose *day 2*). As shown in Fig. 3, *top left*, there was no significant time effect or time × group interaction effect on $k_O - k_D$ values, but a tendency ($P = 0.066$) was observed for a group effect, meaning that $k_O - k_D$ levels tended to be higher in LF-fed mice than in the HF-fed mice.

Analysis of k_D/k_O values revealed a significant group × time interaction [$F_{(4,40)} = 5.88$, $P < 0.001$; Fig. 3, *top right*]. This effect was caused mainly by the difference at the 1st hour, during which the ratio for the HF-fed mice was ~27% lower than at 2 and 3 h. This difference was much smaller in the LF group (at the 1st hour, ~4% lower than at 2 h). There was no significant interaction anymore if 1-h ratio was excluded from the model, indicating no significant difference between 2- and 3-h ratios. Between-group differences persisted over the 3-h period, showing consistently that HF-fed mice overall had a lower k_D/k_O ratio than LF-fed ones, irrespective of weight gain.

Next, we evaluated the effect of the duration of the time interval between initial and final (i.e., taken at 1, 2, and 3 days) on k_O and k_D, and thus the resultant $k_O - k_D$ and k_D/k_O, using one initial time (2 h). In the analysis of $k_O - k_D$ from aforementioned time points, a significant time × group interaction [$F_{(4,40)} = 11.81$, $P < 0.001$] was observed (see Fig. 3, *bottom left*). In both HF-fed groups, $k_O - k_D$ decreased over the course of the 3-day measurement, whereas in LF-fed mice $k_O - k_D$ increased.

Analysis of k_D/k_O showed a group effect [$F_{(2,20)} = 9.34$, $P < 0.01$] and no difference over the 3 days, with LF-fed mice having overall a significantly higher k_D/k_O than the HF-fed DR and DIO mice (Tukey-HSD, $P < 0.01$; see Fig. 3, *bottom right*).

Different methods of calculation. Several equations shown in Table 1 were used to calculate rCO_2, using the 2-h initials. The results, by using the 2-h initals and 2-day finals, are shown in Fig. 4 together with the results of indirect calorimetry. Although the DLW method performed well in the LF group, we observed a significant deviation from the actual rCO_2 based on indirect calorimetry, with DLW systematically overestimating rCO_2 by ~18% (depending on the type of equation and model) in the HF-fed mice irrespectively of body composition. In general, one-pool model equations performed worse than two-pool model ones. Within the DLW results, neither using any of the models nor using intercept or plateau made a significant difference (that is, significant compared with the SE; see Fig. 4). This shows that the DLW method in itself is robust. Nevertheless, it can apparently deliver results that are systematically deviating.

Finally, the multisample approach did not perform better than a two-sample approach when using the initial at 2 h. Differences over 3 days between the two approaches for calculating decay rates were small (<1%).

Duration of the measurement. To evaluate the effect of time between sampling of initial and final enrichments and how this influenced the validity of rCO_2 by DLW analysis (i.e., compared with rCO_2 production assessed by indirect calorimetry), we examined deviations from the latter by using Supplemental Table S1, *Eq. 5*, and the 2-h initial on each day of the DLW
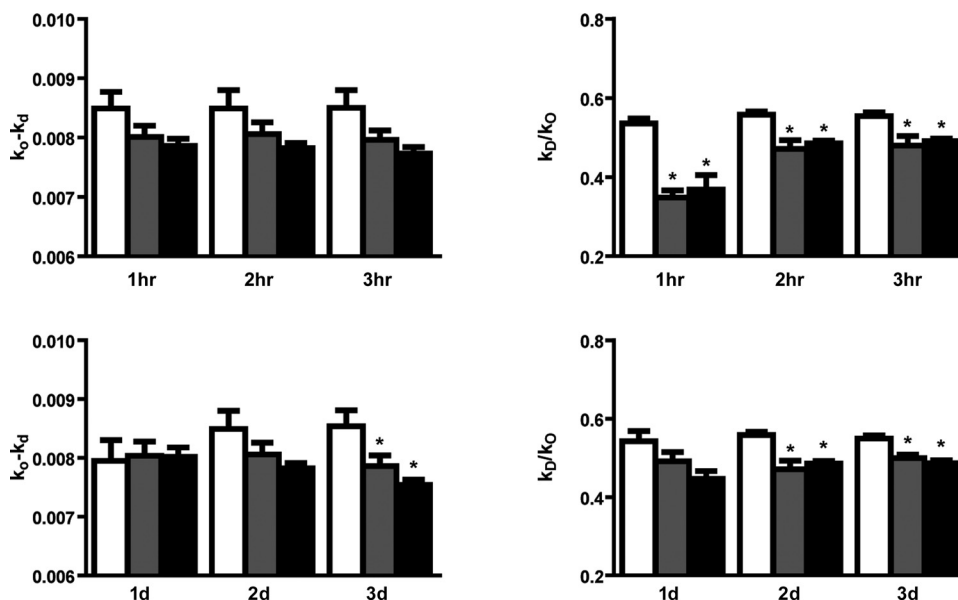

Fig. 3. Comparison of $k_O - k_D$ (*left*) or k_D/k_O (*right*) between having different initials (*top*) and different duration between measurements (*bottom*) in HF-fed DIO (black bar) and DR mice (gray bar) and in LF-fed (open bar) mice. Data are expressed as means ± SE; Tukey's post hoc test. *LF vs. HF-DR or HF-DIO.
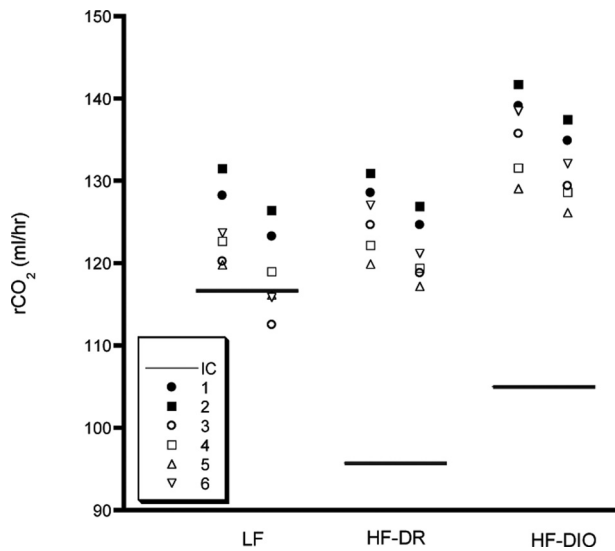
Fig. 4. Mean rate of carbon dioxide production (rCO_2) assessed by indirect calorimetry (horizontal line) and calculated by the doubly labeled water (DLW) method (● and ■, *Eqs. 1* and *2* in Supplemental Table S1 represent 1-pool model equations; ○, □, △, and ▽, *Eqs. 3–6* in Supplemental Table S1 represent 2-pool model equations) for the 3 different groups (LF, HF-DR, and HF-DIO) is presented at *left* in each group when the plateau method was used and at *right* in each group when the intercept was used. The lowest discrepancy was observed in the LF group using Supplemental Table S1, *Eq. 5*, and the plateau method, whereas in the HF group (DIO and DR) the lowest discrepancy was observed using *Eq. 5* Supplemental Table S1 and the intercept method. The results were obtained from the initials taken at 2-h postenrichment and the finals taken 2 days after the initials.

experiment. We used the plateau as well as the intercept method to evaluate body water pool. Results are presented in Table 2. However, the averaged analytical error decreased after each day of measurement (~3% on *day 1*, ~1.5% on *day 2*, and ~1% on *day 3*). When the plateau method was used for estimation of the body water pool, RM-ANOVA showed a significant time × group interaction [$F_{(4,40)} = 8,25$, $P < 001$]. Deviation from rCO_2 assessed by indirect calorimetry increased after each day of measurement in the HF-fed group, whereas it changed within the margin of the analytical error in the LF-fed group. In the LF group, the deviation of estimated rCO_2 from the actual one decreased significantly on *day 3*. When the intercept method was used for estimation of the body water pool, RM-ANOVA showed no significant interaction but a significant effect of time, with the 2nd day of the measurement having relatively higher deviation from indirect calorimetry than at 1 and 3 days.

DISCUSSION

The main aim of this study was to investigate whether nutritional and body composition parameters of mice affect the validity of assessment of rCO_2 by the DLW technique and determine which study design and mathematical model are most optimal. For this purpose, we subjected mice to a HF diet, after which about one half of the cohort developed HF diet-induced obesity (HF-DIO), and the other half was diet-resistant to weight gain (HF-DR) and remained as lean as mice fed a LF diet. By doing so, we were able to dissociate effects of feeding a HF diet on validity of rCO_2 assessment by the DLW method dependent or independent from changes in body composition.

The most important finding here is that an excellent match existed between rCO_2 assessed by the two methods in the LF-feeding mice, whereas there was a consistent overestimation of rCO_2 by the DLW technique in the HF-fed animals compared with actual CO_2 production. This was already anticipated because several other studies in mammals validating the DLW technique reported such overestimation, as reviewed by Speakman and Racey (34) in 1988 and later by others (10, 33). The importance of our study is that feeding a HF diet may be a relevant factor in explaining this inconsistency rather than the increased amount of body fat (or any other compartment associated with this) induced by HF feeding in the DIO-prone mice. DLW studies performed thus far have ignored nutritional or body composition effects on the validity of the rCO_2 assessed by DLW method.

A number of factors may have contributed to this error. When we analyzed the equilibration period during the 3 h after administration of the labels, we observed an incomplete mixing of them, particularly in the HF-fed group, with 1 h being significantly lower in the HF group than in the LF group. It may be possible that the incomplete mixing persisted at 2 and 3 h, which caused overestimation of the body water pool and thus overestimation of rCO_2. In the validation study performed by Schoeller et al. (30), similar incompleteness in the equilibration of the labels was observed in obese human subjects. In that study, in lean subjects a stable equilibration was reached between 2 and 6 h after isotope administration, whereas in obese subjects the estimates were more variable, and the same degree of stability was not obtained before 5 h postdosing.

Verification by carcass analysis through desiccation showed an overestimation of body water by DLW, particularly when the initial at 1 h was used for the calculation of total body water. This difference occurred at 2 and 3 h as well, but to a minor extent. This comparison also showed that the intercept method performed better at longer initial final intervals in determination of body water (from 10 to 5% deviation at 2 and 3 h) than the plateau method.

One possible cause of the overestimation of the body water pool is that hydrogen and/or oxygen can be incorporated into nonaqueous molecules and/or excreted from the body independent of CO_2 and water fluxes. In addition to the increase in body water pool, the dilution space ratio of deuterium and oxygen (N_D/N_O) was lower in the HF-fed mice than in the LF-fed mice. Because N_D/N_O tended to be lower only in the

Table 2. *Percentual deviation of rCO_2 assessed by the DLW method relative to rCO_2 assessed by indirect calorimetry during 3-day measurement, using Eq. 5 in Supplemental Table S1 and the initial taken at 2 h postenrichment, using either the plateau or the intercept method*

	Day 1	Day 2	Day 3
Plateau			
LF	−0.5 ± 1.1	3.9 ± 1.5	−5.7 ± 2.4
HF-DR	19.5 ± 2.9	25.8 ± 2.8	39.6 ± 6.2
HF-DIO	21.3 ± 3.3	23 ± 2.1	33.0 ± 4.4
Intercept			
LF	−3.3 ± 1.0	0.8 ± 1.5	−1.4 ± 1.8
HF-DR	16.7 ± 2.7	23.0 ± 2.7	18.9 ± 2.1
HF-DIO	18.7 ± 3.2	20.2 ± 2.1	17.0 ± 1.7

Data are expressed as means ± SE. DLW, doubly labeled water.

HF-DIO vs. the HF-DR mice, this points toward primary dietary effects on N_D/N_O, but body composition effects may be not ruled out. Thus, the estimated turnover of hydrogen was too low, the estimated turnover of oxygen was too high, or both processes occurred simultaneously. One potential mechanism is that the HF-fed mice have a lower level of hydrogen and methane production by gut microbiota as a result of lower dietary fiber density than the LF-fed mice, which is analogous to what has been found in humans and pigs (13, 22). In turn, this then would lead to a relatively lower ^2H turnover in the HF-fed mice than in the LF-fed mice. Another potential mechanism contributing to fluxes independent from water and CO_2 is an elevated level of ketone body production by HF feeding. Normally, ketone bodies are produced during fasting as a side effect of β-oxidation, which can be used as an alternative substrate in the brain. Several studies have shown that the feeding of a HF diet will also lead to an upregulation of the production of ketone bodies (5, 6, 15, 25), and when not sufficiently metabolized they will accumulate in the urine or breath because these fuels are water soluble (6). The net loss of ketone bodies will constitute an increased ^{18}O flux from the body independent of CO_2 and water turnover. Of course ^2H is dragged along in this process, but in a smaller proportion to ^{18}O in ketones compared with water. ^{18}O and ^2H fluxes could also be increased due to incorporation of these molecules in urea via the ornithine-arginine synthesis cycle. This effect may be substantial if the HF-fed mice have an increased extent of protein degradation and urea synthesis (7, 35). At present, it is extremely difficult to make summated numerical estimates to the extent by which all of these pathways contribute to ^{18}O and ^2H turnovers beyond those mediated by CO_2 and water turnover. A final, even more exotic explanation of which very little is known is that isotopic levels of H and O atoms in intracellular water could differ from the extracellular compartment by physiological processes that discriminate between isotopes (16, 17). The latter processes could potentially explain differential intra/extracellular isotopic composition and could be underlying to the differences in the N_D/N_O ratio in the HF-fed mice relative to the LF-fed mice.

In summary, our data showed that, compared with actual rCO_2, the DLW method performed extremely well when rCO_2 in the LF-fed mice was assessed with a precision of -0.5 ± 1.1 (considering an analytical error of ~1.5%). Furthermore, the intercept method appears to perform superiorly over the plateau method in estimating isotopic pool sizes, in particular for longer measurement duration (i.e., 3 days). We did not observe a significant improvement in the multisample approach over the two-sample one. We recommend particular caution with the use of the DLW technique in HF-fed humans and animals because it may overestimate MR calculated by DLW. These findings can have major consequences for the interpretation of results obtained by the DLW technique, particularly in obesity research. Future research should be dedicated to the mechanisms (of which potentially some are mentioned above) underlying this discrepancy and how to correct for it.

AUTHOR CONTRIBUTIONS

S.G., H.A.J.M., and G.v.D. contributed to the conception and design of the research; S.G. performed the experiments; S.G. analyzed the data; S.G. and G.v.D. interpreted the results of the experiments; S.G. prepared the figures; S.G. drafted the manuscript; S.G., H.A.J.M., and G.v.D. edited and revised the manuscript; S.G., H.A.J.M., and G.v.D. approved the final version of the manuscript.

REFERENCES

1. **Barf RP, Van Dijk G, Scheurink AJ, Hoffmann K, Novati A, Hulshof HJ, Fuchs E, Meerlo P.** Metabolic consequences of chronic sleep restriction in rats: changes in body weight regulation and energy expenditure. *Physiol Behav* 107: 322–328, 2012.
2. **Bergmann BM, Everson CA, Kushida CA, Fang VS, Leitch CA, Schoeller DA, Refetoff S, Rechtschaffen A.** Sleep deprivation in the rat: V. Energy use and mediation. *Sleep* 12: 31–41, 1989.
3. **Bryce JM, Speakman JR, Johnson PJ, Macdonald DW.** Competition between Eurasian red and introduced Eastern grey squirrels: the energetic significance of body-mass differences. *Proc Biol Sci* 268: 1731–1736, 2001.
4. **Coward WA, Prentice AM.** Isotope method for the measurement of carbon dioxide production rate in man. *Am J Clin Nutr* 41: 659–663, 1985.
5. **Dierks-Ventling C, Cone AL.** Ketone body enzymes in mammalian tissues. Effect of high fat diet. *J Biol Chem* 246: 5533–5534, 1971.
6. **Galvin RD, Harris JA, Johnson RE.** Urinary excretion of beta-hydroxy-butyrate and acetoacetate during experimental ketosis. *Exp Physiol* 53: 181–193, 1968.
7. **Gauthier MS, Couturier K, Charbonneau A, Lavoie JM.** Effects of introducing physical training in the course of a 16-week high-fat diet regimen on hepatic steatosis, adipose tissue fat accumulation, and plasma lipid profile. *Int J Obes Relat Metab Disord* 28: 1064–1071, 2004.
8. **Gehre M, Geilmann H, Richter J, Werner RA, Brand WA.** Continuous flow 2H/1H and 18O/16O analysis of water samples with dual inlet precision. *Rapid Commun Mass Spectrom* 18: 2650–2660, 2004.
9. **Guidotti S, Jansen HG, Aerts-Bijma AT, Verstappen-Dumoulin BM, van Dijk G, Meijer HA.** Doubly Labelled Water analysis: preparation, memory correction, calibration and quality assurance for δ2H and δ18O measurements over four orders of magnitudes. *Rapid Commun Mass Spectrom* 27: 1055–1066, 2013.
10. **Haggarty P, Franklin MF, Fuller MF, McGaw BA, Milne E, Duncan G, Christie SL, Smith JS.** Validation of the doubly labeled water method in growing pigs. *Am J Physiol Regul Integr Comp Physiol* 267: R1574–R1588, 1994.
11. **Haggarty P, McGaw BA, Franklin MF.** Measurement of fractionated water loss and CO2 production using triply labelled water. *J Theor Biol* 134: 291–308, 1988.
12. **Hellerstein MK.** Synthesis of fat in response to alterations in diet: Insights from new stable isotope methodologies. *Lipids* 31: S117–S125, 1996.
13. **Jensen BB, Jørgensen H.** Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. *Appl Environ Microbiol* 60: 1897–1904, 1994.
14. **Kerstel ER, Piersma T, Gessaman JA, Dekinga A, Meijer HA, Visser GH.** Assessment of the amount of body water in the Red Knot (Calidris canutus): an evaluation of the principle of isotope dilution with 2H, (17)O, and (18)O as measured with laser spectrometry and isotope ratio mass spectrometry. *Isotopes Environ Health Stud* 42: 1–7, 2006.
15. **Krebs HA, Hems R.** Fatty acid metabolism in the perfused rat liver. *Biochem J* 119: 525–533, 1970.
16. **Kreuzer-Martin HW, Lott MJ, Ehleringer JR, Hegg EL.** Metabolic processes account for the majority of the intracellular water in log-phase Escherichia coli cells as revealed by hydrogen isotopes. *Biochemistry* 45: 13622–13630, 2006.
17. **Kreuzer HW, Quaroni L, Podlesak DW, Zlateva T, Bollinger N, McAllister A, Lott MJ, Hegg EL.** Detection of metabolic fluxes of O and H atoms into intracellular water in mammalian cells. *PLoS One*. 7: e39685, 2012.
18. **Krol E, Speakman JR.** Isotope dilution spaces of mice injected simultaneously with deuterium, tritium and oxygen-18. *J Exp Biol* 202: 2839–2849, 1999.

19. **Kronfeld-Schor N, Shargal E, Haim A, Dayan T, Zisapel N, Held-maier G.** Temporal partitioning among diurnally and nocturnally active desert spiny mice: energy and water turnover costs. *J Therm Biol* 26: 139–142, 2001.

20. **Lifson N, Gordon GB, McClintock R.** Measurement of total carbon dioxide production by means of D2O18. *J Appl Physiol* 7: 704–710, 1955.

21. **Lifson N, McClintock R.** Theory of use of the turnover rates of body water for measuring energy and material balance. *J Theor Biol* 12: 46–74, 1966.

22. **Marthinsen D, Fleming SE.** Excretion of breath and flatus gases by humans consuming high-fiber diets. *J Nutr* 112: 1133–1143, 1982.

23. **McClintock R, Lifson N.** Applicability of the D2O18 method to the measurement of the total carbon dioxide output of obese mice. *J Biol Chem* 226: 153–156, 1957.

24. **Nagy KA.** CO_2 production in animals: analysis of potential errors in the doubly labeled water method. *Am J Physiol Regul Integr Comp Physiol* 238: R466–R473, 1980.

25. **Oussadou L, Griffaton G, Kalopissis AD.** Hepatic VLDL secretion of genetically obese Zucker rats is inhibited by a high-fat diet. *Am J Physiol Endocrinol Metab* 271: E952–E964, 1996.

26. **Racette SB, Schoeller DA, Luke AH, Shay K, Hnilicka J, Kushner RF.** Relative dilution spaces of ^{2}H- and ^{18}O-labeled water in humans. *Am J Physiol Endocrinol Metab* 267: E585–E590, 1994.

27. **Schoeller DA.** Measurement of energy expenditure in free-living humans by using doubly labeled water. *J Nutr* 118: 1278–1289, 1988.

28. **Schoeller DA, Leitch CA, Brown C.** Doubly labeled water method: in vivo oxygen and hydrogen isotope fractionation. *Am J Physiol Regul Integr Comp Physiol* 251: R1137–R1143, 1986.

29. **Schoeller DA, Ravussin E, Schutz Y, Acheson KJ, Baertschi P, Jé-quier E.** Energy expenditure by doubly labeled water: validation in humans and proposed calculation. *Am J Physiol Regul Integr Comp Physiol* 250: R823–R830, 1986.

30. **Schoeller DA, van Santen E, Peterson DW, Dietz W, Jaspan J, Klein PD.** Total body water measurement in humans with 18O and 2H labeled water. *Am J Clin Nutr* 33: 2686–2693, 1980.

31. **Speakman JR.** Assumptions in the simple model of Lifson and co-workers. In: *Doubly Labelled Water: Theory and Practice*. London: Chapman & Hall, 1997, chapt. 7, p. 81–176.

32. **Speakman JR, Nair KS, Goran MI.** Revised equations for calculating CO2 production from doubly labeled water in humans. *Am J Clin Nutr* 264: E912–E917, 1993.

33. **Speakman JR, Perez-Camargo G, McCappin T, Frankel T, Thomson P, Legrand-Defretin V.** Validation of the doubly-labelled water technique in the domestic dog (Canis familiaris). *Br J Nutr* 85: 75–87, 2001.

34. **Speakman JR, Racey PA.** Validation of the doubly labeled water technique in small insectivorous bats by comparison with indirect calorimetry. *Physiol Zool* 514–526, 1988.

35. **Tarnawski R, Tomasik A.** Changes of the activities of urea cycle enzymes extracted from rat liver after surgical reduction of stomach capacity and after feeding a high-fat diet. *Arch Tierernahr* 46: 165–171, 1994.

36. **Turner SM, Murphy EJ, Neese RA, Antelo F, Thomas T, Agarwal A, Go C, Hellerstein MK.** Measurement of TG synthesis and turnover in vivo by ^{2}H$_2$O incorporation into the glycerol moiety and application of MIDA. *Am J Physiol Endocrinol Metab* 285: E790–E803, 2003.

37. **Visser GH, Boon PE, Meijer HA.** Validation of the doubly labeled water method in Japanese Quail Coturnix c. japonica chicks: is there an effect of growth rate? *J Comp Physiol B* 170: 365–372, 2000.

38. **Weir JB.** New methods for calculating metabolic rate with special reference to protein metabolism. *Nutrition* 6: 213–221, 1990.

39. **Wikelski M, Tarlow EM, Raim A, Diehl RH, Larkin RP, Visser GH.** Costs of migration in free-flying songbirds. *Nature* 423: 2003.