Validity of Triply Labelled Water Analysis for Energy Expenditure Measurements in Mice

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Validity of Triply Labelled Water analysis for energy expenditure measurements in mice

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Abstract

The Doubly Labelled Water (DLW) method is widely used to determine energy expenditure. In this work, we demonstrate the addition of the third stable isotope, ^{17}O, to turn it into Triply Labelled Water (TLW), using the three isotopes measurement of optical spectrometry. We performed TLW (^{2}H, ^{18}O and ^{17}O) measurements for the analysis of the CO_2 production (r_{CO2}) of mice on different diets for the first time. Triply highly enriched water was injected into mice, and the isotope enrichments of the distilled blood samples of one initial and two finals were measured by an Off-Axis Integrated Cavity Output Spectroscopy instrument. We evaluated the impact of different calculation protocols and the values of evaporative water loss fraction. We found that the dilution space and turnover rates of ^{17}O and ^{18}O were equal for the same mice group, and that values of r_{CO2} calculated based on ^{18}O-^{2}H, or on ^{17}O-^{2}H agreed very well. This increases the reliability and redundancy of the measurements and it lowers the uncertainty in the calculated r_{CO2} to 3% when taking the average of two DLW methods. However, the TLW method overestimated the r_{CO2} compared to the indirect calorimetry measurements that we also performed, much more for the mice on a high-fat diet than for low-fat. We hypothesize an extra loss or exchange mechanism with a high fractionation for ^{2}H to explain this difference.

Introduction

The Doubly Labelled Water (DLW) method, first proposed by Lifson et al. in the middle of the last century, is a reliable, harmless and non-invasive method to determine the energy expenditure and body composition for humans and free-living animals [1–6]. In practice, a dose of known concentration water, highly enriched in the isotopes ^{2}H and ^{18}O, is introduced to the body, where the mixtures will quickly spread evenly through the body water pool and thus get diluted. The general principle of the method is based on the fact that hydrogen leaves the body through water turnover, while oxygen leaves both through water turnover and through respiratory CO_2. The ^{2}H and ^{18}O abundances of body fluids are measured from the initial to the final time points, and then the isotope elimination rates can be determined. The difference between these two turnover rates is then proportional to the CO_2 production (r_{CO2}), which can be further converted to energy expenditure if the composition of the food intake is known [1,4,7–9].

Although the basic theory is straightforward, several complications are involved when conducting the actual r_{CO2} calculation [2,4,10–14]. For example, we need to consider the oxygen isotope fractionation between CO_2 and body water, as well as the fractionation between water and water vapour. These fractionation factors are well known from various laboratory experiments in the past [4,8,15–19]. However, certain aspects of the process are less well-known (and possibly variable), such as the level of (non-)equilibrium in the fractionation process during evaporative H_2O loss, and the fraction of the water that leaves the body through evaporation. Different approaches for body water pool size calculations also matter for the final results [13,20], although one can argue that the choice for the optimal way of calculation is clear from a principle point of view.
In the history of DLW, a series of calculation protocols have been used and studied. The comparison with other methods estimating energy expenditure methods, like the indirect calorimetry method, is highly valuable [13,21–23].

In addition to the traditional DLW method, some researchers proposed or even used three isotopes instead of two (the combination of ^{2}H, ^{3}H and ^{18}O or ^{2}H, ^{17}O and ^{18}O), to trace isotope changes for quantifying the body water and CO_2 fluxes [10,16,24]. The additional use of the third isotope reduces the analytical error, helps to check the data quality and in principle even gives the possibility to quantify the evaporative water loss fraction. However, ^{3}H (tritium) is rare and radioactive, and therefore not attractive to complement ^{2}H and ^{18}O as a tracer. Addition of the other naturally occurring rare stable isotope of oxygen, ^{17}O, was for a long time unattractive due to the complicated measurement methods when using Isotope Ratio Mass Spectrometry (IRMS), due to the mass overlap of the $^{12}C^{17}O^{16}O$ and the $^{13}C^{16}O^{16}O$ isotopologues. Avoiding this overlap could either be done by reduction of CO_2 to O_2 by fluorination [25,26] or by direct water electrolysis [27]. In connection to the complicated measurements, enriched ^{17}O water is rather expensive for application to DLW studies due to almost absent demand.

For a long time, IRMS has been the technique for DLW water analysis. As IRMS functions with pure gases, pre-treatment for the (water) samples is necessary, preceded by distillation if the body fluid is blood [2,4,9,28]. Optical spectroscopic measurement of water vapour has become a reliable alternative, initially thanks to the pioneering activities in our laboratory [14,29,30]. At present, there is commercial equipment available, which enables the measurement of the isotope ratios for the DLW method faster and easier, but with equivalent precision and accuracy compared with IRMS [20,31–34]. The advantage of optical spectrometry is that all water

isotopologues can be measured independently, so the simultaneous analysis of ^{2}H, ^{17}O and ^{18}O of water samples and biological fluids is possible. This provides the possibility to add the third isotope, ^{17}O, to DLW analysis, and turn it into Triply Labelled Water (TLW).

In this study, we make use of this possibility, and demonstrate, to our knowledge for the first time, complete TLW measurements for the analysis of the CO_2 production of mice in different diet types. Triply highly enriched ^{2}H, ^{17}O and ^{18}O water was injected into mice, and isotope enrichment of the distilled blood samples were measured by optical spectrometry, using available reference waters of ^{2}H and ^{18}O, and home-made ^{17}O reference waters. We describe how we conduct the TLW method and give several calculation protocols. Then we analyze the advantage of the TLW method, the difference of calculation protocols, the deviation of CO_2 production measured by TLW and indirect calorimetry, and the influence of different nutrition for mice. The last step, converting the produced CO_2 to energy expenditure, is a mere multiplication by the energy equivalent value for the food. Since this study focusses on method evaluation, we refrain from this step and stick with the produced CO_2.

Material and methods

Animals and housing

All experimental procedures involving animals were approved by the local Animal Experimentation Committee (DEC) of the University of Groningen (protocol number: 198664-01-001), and guided by Dutch Animal Experimental Committee in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), as well as the ARRIVE 2.0 guidelines. Twenty male C57BL6/J mice were individually housed on a 12:12 light-dark cycle with food and water ad libitum, and a controlled temperature (22 ± 1°C) (more details in [13]). At the age of 27 weeks, ten of the mice were maintained on regular chow diet, the so-called low-fat diet (LF) group (17.5 kJ/g; fat content 13.5 %; protein content 28 %; carbohydrate content 58 %). The other ten mice were changed to a high-fat sucrose diet (HF) (21.8 kJ/g; fat content 28 %; protein content 19.5 %; carbohydrate content 52.5 %) eleven weeks prior to the TLW injection.

Preparation of the Triply Labelled Water

We produced a highly enriched TLW mixture by mixing the ^{2}H, ^{18}O and ^{17}O "mother" waters (around 8.0, 12.4 and 6.2 grams, respectively, determined with 0.1 mg precision). The "mother" waters are purely ^{2}H water ($[^{2}H] > 99.9$ %, Sigma-Aldrich, Netherlands), ^{18}O water ($[^{18}O] \approx 98$ %, ROTEM industries, Rehovoth, Israel) and ^{17}O water with high enrichment levels ($[^{17}O] \approx 41$ %, $[^{18}O] \approx 43$ %, ROTEM industries, Rehovoth, Israel). This resulted in a mixture ($[^{2}H] = 29.7$ %, $[^{18}O] = 55.88$ %, $[^{17}O] = 8.55$ %). This is equivalent to enrichment factors of ≈ 1900, 280 and 225, respectively, so in our experiments we expect higher enrichments for $\delta^{2}H$ than for $\delta^{18}O$ and $\delta^{17}O$, whereas the latter two will be roughly equal. Given the measurement uncertainty of our Off-Axis Integrated Cavity Output Spectroscopy (OA-ICOS) analyzer, we expect similar accuracies in the measurements for all three isotopes this way.

Using this mixture for the injection in the mice, we estimated that a 0.17 g injection would result in the initial samples (the most enriched ones) having δ^2H and $\delta^{18}O$ values close to the international enriched reference water IAEA-609 (δ^2H = 16036.4 ‰, $\delta^{18}O$ = 1963.7 ‰ [35]). Therefore, the suite of enriched water references IAEA-609,608 and 607 are suitable for δ^2H and $\delta^{18}O$ calibration of all mice blood samples. However, as these reference waters are not (or only mildly) enriched in ^{17}O, we cannot use them for the calibration of our $\delta^{17}O$ measurements, where we expect initial values of around 1700 ‰. Therefore, to calibrate TLW measurements, we made a range of four reference waters enriched in all three isotopes, by gravimetrically mixing the highly enriched TLW mixture ([^2H] = 29.7 %, [^{18}O] = 55.88 %, [^{17}O] = 8.55 %) with varying quantities of demineralized tap water (δ^2H = -42.49 ‰, $\delta^{18}O$ = -6.36 ‰, $\delta^{17}O$ = -3.39 ‰). Different amounts of the TLW mixture, from 0.25 to 0.6 g (~0.1 mg precision), were put into a 2 ml glass vial, and then immersed into a glass bottle which contains about 100 g demineralized water (~0.1 mg precision). These bottles were sealed after mixing and shaken periodically for several hours.

Table 1 shows the values of these four TLW-references along with their uncertainty. The δ^2H and $\delta^{18}O$ values were measured by OA-ICOS and calibrated using IAEA-609, 608 and 607. The error of δ^2H is based on several measurement repetitions, but for $\delta^{18}O$, the measurement uncertainty is small (less than 1 ‰), so the uncertainty of IAEA waters are important for the $\delta^{18}O$ error of these TLW-references [35].

[Table 1 is here**]**

The IAEA waters are unfortunately only mildly enriched in ^{17}O, so for the $\delta^{17}O$ value determination of our four TLW reference waters, we conducted several dilution experiments to bring the resulting $\delta^{17}O$ values of the diluted TLW reference waters within the range of the IAEA waters (with IAEA-609 having the maximum $\delta^{17}O$ value of 126.6 ‰). By using the accurately determined dilution factor, we could in this way calibrate the $\delta^{17}O$ of TLW reference waters using IAEA-609,608 and 607. All calculations of isotope abundances and δ-values were performed using a thoroughly validated Excel spreadsheet [36]. Based on the measurement uncertainties, the uncertainties in the values quoted for the IAEA waters, and the dilution uncertainties we attribute a -conservative- ± 1 % relative uncertainty to our $\delta^{17}O$ values (see table 1). Besides the best estimates for the $\delta^{17}O$ of each TLW reference water, we also could determine the abundances of the highly enriched TLW ([^2H] = 29.7 %, [^{18}O] = 55.88 %, [^{17}O] = 8.55 %). We separately determined that the ^{18}O mother water has a 98.42 % abundance, deviating somewhat from 98.2 % provided by the manufacturer (but within their specification). The ^{17}O mother water contains 34.47 % [^{17}O] and 42.65 % [^{18}O] (for [^{17}O] deviating from the manufacturer's specification of 41.1 % whereas [^{18}O] with 43 % agrees). The ^2H mother water is virtually pure. The enriched reference waters and the highly enriched TLW mixtures were stored in thoroughly closed bottles inside a sealed container, filled with dry N_2 gas at slight overpressure. This prevented the uptake of -and thus dilution by- atmospheric moisture.

Experimental design

For the experiments, each mouse was intraperitoneally injected with about 0.17 g (weighted to the nearest 0.1 mg) of the highly enriched TLW mixture. Before injection, we took 4 blood

samples separately from 5 mice for background isotope analysis. After exactly two hours after the TLW injection, the initial blood samples were taken. Then, the mice were transferred to the indirect calorimetry (IC) cages for two consecutive days. The indirect calorimetry (IC) was shortly interrupted at exactly (deviations less than 2 minutes) 24 and 48 hours after the initial sample time. In this study, the background and TLW mixture blood were all sampled by tail snip (4 times per sample every time), and then flame-sealed into 25 µl glass capillaries until the micro-distillation process [13]. Mice body mass were measured by a balance (~0.1 g precision), and fat, lean weight and water content of all the mice were measured by a magnetic resonance imaging machine (EchoMRI) just before injection [37].

Indirect calorimetry

In the indirect calorimeter (IC) cages, the housing and feeding conditions were not changed. The detailed description of the IC method in our lab is in [13]. In brief, the IC system measured the O_2 and CO_2 concentration difference of the dried inlet air (reference) and dried outlet air going through the chambers. The flow rate of the inlet was set at 20 l/h, and only 6 l/h outlet air passed through the drying system and subsequently to the gas analyzer. The mass-flow controllers were calibrated before and after the trials (the variation < 1 %). O_2 was measured by a paramagnetic O_2 analyzer (Sevomex Xentra 4100, Crowborough, UK), and CO_2 by an infrared gas analyzer (Servomex 1440). The CO_2 analyzer was calibrated daily with certified gas standards, and the maximum overall error of the method is ≤ 2 %. Comparable methods were applied for O_2 calibration. For validation purposes, the respiratory quotients ($RQ = r_{CO2} / r_{O2}$) and metabolic rates (MR) were also recorded and calculated [13,38].

Analysis method of the TLW samples

The δ^2H, $\delta^{17}O$ and $\delta^{18}O$ of mice blood samples and reference waters were measured by a commercial Off-Axis Integrated Cavity Output Spectroscopy (OA-ICOS) Liquid Water Isotope Analyser (LWIA 912-0050, ABB-Los Gatos Research, San Jose, CA, USA). Before injection into the analyser, all the samples and references were prepared by a home-built micro-distillation system (detailed distillation procedures are described in [39]). In brief, a capillary is broken in an evacuated system, and the water is collected in a freeze finger immersed in liquid nitrogen. The system is then again evacuated, and the water is finally transferred, again using liquid nitrogen, into a small insert tube, which can be measured directly on the OA-ICOS analyser. The reference waters (the IAEA series and our TLW references) are treated identically, so also transferred from capillaries.

The distilled samples and references were introduced into the OA-ICOS instrument through an auto-injector (CTC Pal), and there is a heated injector block to evaporate the liquid water. This vapour expands into a high-finesse optical cavity, and the δ^2H, $\delta^{18}O$ and $\delta^{17}O$ values were calculated from fits to the relative transmission spectrum. The distilled IAEA-609,608 and 607 and our local TLW references are interleaved with samples during the measurement series for calibration and instrumental drift correction. Each reference and sample water was injected 12 times. Before each distillate reference, the same reference water, but without distillation, is also injected 12 times to check the micro-distillation quality and stability, and also to reduce memory effects. However, only the distilled references were used for calibration.

Raw data from the instrument were analysed using a bespoke data analysis program (written in R), through which memory effects and drifts were corrected, and calibration was performed (for details see [40]). Specifically, to correct for memory effects we do not ignore the first few injections of a sample, but instead use all of them and correct for the memory effects using a 2-3 pool exchange algorithm [39]. This is quite meaningful for TLW blood samples, which have a minimal sample size (less than 15 µl). Figure 1 shows a representative part of a measurement batch (containing "initial" samples), in which both the raw, and memory corrected values for several samples and reference waters are shown. The improvement in precision is remarkable: standard deviations of the 12 δ^2H measurements of the samples around 6000 ‰, for example, reduce from 210 ‰ to 30 ‰ when measured just after natural (demineralized Groningen tap-) water (δ^2H ≈ -42 ‰).

[Fig 1. is here**]**

For calibration, mostly a “multiple-point” quadratic fit is chosen, which is based on three or more of the reference waters. This is based on our experience that for these highly enriched samples and the large range in δ-values in each series (eg: δ^{18}O from 736 ‰ to 1963 ‰ for the IAEA reference waters), the instrument's output is not fully linear. This is probably due to imperfect line fitting, which also makes itself noticeable through relatively high values for the so-called "Narrow Band" spectroscopic interference [41]. This tool is meant to be an indicator for contamination, but as contamination does not occur in our samples (and certainly not in the pure reference waters), here it is the result of an imperfection in the spectroscopic fit of these triply labelled waters.

As illustrated in [39], duplicate analysis of DLW samples is necessary and helpful, due to the dominant uncertainty contribution of the actual procedure of flame-sealing and micro-distillation. In this study, if the δ^2H value of a duplicate analysis deviates more than 2 % of its value from the first (or 1.5 % for δ^{18}O, 1.5 % for δ^{17}O), a third sample is analyzed. A third sample is also taken if the quality of a capillary is questionable (for example not tight or containing too much air). The average of the duplicate (or triplicate if an outlier cannot be identified) analyses, along with the standard error in the mean is taken as the final result. The OA-ICOS measurement uncertainty for individual samples is usually negligibly small compared to the spread between duplicate samples.

For the TLW method analysis, we use isotope abundances instead of the δ-values. First, the sample’s ${}^x\delta_s$ values need to be converted into abundance ratios ${}^xR_s$ (x = 2, 17 and 18), using the isotope abundance ratios for Vienna Standard Mean Ocean Water (VSMOW), which are 1.5576×10^{-4}, 3.799×10^{-4}, and 2.0052×10^{-3} for ^{2}H, ^{17}O and ^{18}O, respectively [42]:

$$ {}^xR_s = {}^xR_{VSMOW} \times (1 + {}^x\delta_s) \quad (1) $$

From these ratios, the absolute isotope concentrations ${}^xC_s$ are computed, usually expressed in parts per million (ppm):

$$ {}^2C_s = \frac{{}^2R_s}{1 + {}^2R_s} \quad (2) $$

$$^{18}C_s = \frac{^{18}R_s}{1 + {}^{18}R_s + {}^{17}R_s} \quad (3)$$

$$^{17}C_s = \frac{^{17}R_s}{1 + {}^{18}R_s + {}^{17}R_s} \quad (4)$$

Calculations

After the injection of the enriched TLW mixtures into the mice, the enriched rare isotopes are gradually exchanged with the surroundings, and the turnover rate (k; h^{-1}) describing the rare isotope concentration decrease can be expressed as:

$$k_t = \frac{ln\left[\frac{(C_i - C_b)}{(C_f - C_b)}\right]}{t} \quad (5)$$

C is the concentration of the isotope ^2H, ^{18}O or ^{17}O. "i" means the initial, and in this study, the initial sample is the 2-hour samples taken after injection. "b" is background (concentrations corresponding to δ^2H = -27.3 ‰, $\delta^{18}O$ = -4.85 ‰, $\delta^{17}O$ = -2.61 ‰, as established based on sampling five mice prior to the injection of TLW), and "f" is the the final sample (taken either 24 hours or 48 hours after the initial sample), therefore, time duration "t" is equal to 24 or 48 hours.

The dilution space of the isotopes in the body, and thus the size of the body water pool, can be calculated using the measurement of the initial concentration by the so-called plateau method [4]:

$$N = Mol_{inj}\frac{C_i - C_{inj}}{C_b - C_i} \quad (6)$$

where N (mol) represents the dilution space or body water pool for ^2H (N_{2H}), ^{18}O (N_{18O}) and ^{17}O (N_{17O}). Mol_{inj} is the number of the moles of the injection TLW (19.81 g/mol) and C_{inj} is the injected enrichment ([^2H] = 29.7 %, [^{18}O] = 55.88 %, [^{17}O] = 8.55 %). In this expression, the loss of enriched isotopes in the two hours between the injection and the initial measurement is ignored. Alternatively, one can take this loss into account by extrapolating the turnover rate back to the injection time. This is called the intercept method [4]:

$$N = Mol_{inj}\frac{C_{i-ic} - C_{inj}}{C_b - C_{i-ic}} \quad (7)$$

$$C_{i-ic} = (C_i - C_b)e^{k_i(t_i - t_{inj})} + C_b \quad (8)$$

where $C_{i\text{-}ic}$ is the concentration extrapolated back to the time of injection (0 hour), and t_i-t_{inj} is equal to 2 hours in our case.

Whereas the plateau method is expected to underestimate the body water pool slightly (as the loss of enriched isotopes during the first two hours is ignored), the intercept method, on the other hand, possibly overestimates the body water pool, as the loss of enriched isotopes during

the first two hours is probably less than later, since the enriched isotopes have not distributed themselves over the entire body water pool. Therefore, calculating and comparing both is a good practice.

It is generally observed that the body water pool as determined by ^{2}H is slightly, but significantly, larger than that by ^{18}O [4,13,43]. This is commonly attributed to the exchange of hydrogen (and thus ^{2}H) with body tissues, which does not occur with oxygen. For this reason, we expect the body water pool determination using ^{17}O to be identical to that with ^{18}O.

The amount of total body water (TBW, g) for each individual animal is then simply:

$$TBW = M \times N \tag{9}$$

M is the molar mass of water (18.02 g/mol). In terms of carbon dioxide production, in a simple expression ignoring the fractionation effects, the difference between ^{2}H and ^{18}O turnover is proportional to the rate of CO_2 production (rCO_2 ; mol/h):

$$r_{CO2} = \frac{N}{2} \times (k_{18O} - k_{2H}) \tag{10}$$

Also here, several fractionation effects occur in the process. Therefore, this equation (10) is not suitable for an accurate calculation of rCO_2. However, as the deviations are relatively small, this equation can be used for uncertainty propagation calculations. The full expression contains the following fractionation factors: the (partly kinetic, partly equilibrium) evaporation of water for ^{2}H (f_1) and ^{18}O ($f_{2,18O}$), and the CO_2-H_2O fractionation for ^{18}O ($f_{3,18O}$), which is assumed to be in equilibrium:

$$r_{CO2} = \frac{N}{2 \times f_{3,18}} \times (k_{18O} - k_{2H}) - r_G \times \frac{f_{2,18O} - f_1}{2 \times f_{3,18O}} \times N \times k_{2H} \tag{11}$$

r_G is the fraction of the water loss due to evaporation, as it happens in the lungs. By lack of a firm determination or estimate, most studies use a value of 0.5. The isotopic fractionation process leads to relatively lower abundances of the heavy isotopes in the vapour phase. All fractionation factors are shown in table 2, and equation (11) is from [4]. If instead of on ^{18}O and ^{2}H turnover, rCO_2 is calculated based on the ^{17}O and ^{2}H turnover, we arrive at the identical equation, but with the ^{17}O decay rate, and two fractionation factors now for ^{17}O:

$$r'_{CO2} = \frac{N}{2 \times f_{3,17O}} \times (k_{17O} - k_{2H}) - r_G \times \frac{f_{2,17O} - f_1}{2 \times f_{3,17O}} \times N \times k_{2H} \tag{12}$$

where k_{17} is the turnover rate for ^{17}O, and $f_{2,17O}$ and $f_{3,17O}$ are the fractionation factors for ^{17}O fractionation in the water evaporation and the CO_2-H_2O equilibrium, respectively.

In table 2, we list the fractionation factors obtained from literature, as well as the 'mixed' results by the equilibrium/kinetic as a ratio of 3:1 [4], and the final $(f_2 - f_1)/2f_3$ calculation results. All the factors are equal to the values listed in [16] and [4], expect the $f_{3,17}$. Its value of 1.0202 is obtained based on the equation from [17] at 37°C, and $\ln(\alpha 17)/\ln(\alpha 18) = 0.5229$ [44].

[Table 2 is here**]**

There are several classical equations to calculate the CO_2 production, which differ in the selection of fractionation factor values, portion of fractionation water (r_G) and body water pool models, and are also dependent on the research subjects (animals or humans) [4,16,45–47]. Equation (11) and (12) use a single pool model, they are reproduced as equations1-1 and 1-2 in Table 3. For ^{18}O, equation 1-1 is similar to the expression in [4] except the number of decimal places, and equation 1-2 is for ^{17}O based on the same calculation principle. When we consider the two-pools model, which means that the effective body water pool is taken differently for ^{2}H than for ^{18}O (or ^{17}O), the Coward 1985 [46] and Speakman 1993 [45] models are more logical and suitable for animal CO_2 production calculation. The equations 2-1, 2-1, 3-1, 3-2 in table 3 are based on their model principle, separately from Coward 1985 [46] and Speakman 1993 [45]. The fractionation factors (from Table 2) used for the equations in Table 3 are the same, irrespective of the model. The R_{dil} in 3-1 and 3-2 is the mean dilution space ratio N_{2H}/N_O (the dilution space calculated by ^{2}H divided by the dilution space calculated by ^{17}O or ^{18}O) for different group members, so different for the low and high fat diet mice.

[Table 3 is here**]**

Results

Body composition

After 11 weeks on a high-fat diet, the high-fat diet mice gained more than 5 grams of weight. On the basis of the body mass gain (> 10 g or < 10 g), 5 mice were assigned to be obesity-resistant (HF-OR), and 5 mice were in the obesity-prone (HF-OP) group. The body mass weighed just before injection were used as body weight, together with the fat mass, lean mass, and body water measurement by EchoMRI. From the 10 mice on low fat diet (LF), two had to be discarded from the data set due to blood sampling problems (only one successful capillary for the initial sampling, and large discrepancies between their calculated total body water by TLW and by EchoMRI).

Figure 2 illustrates the average values of the body-weight, lean content (gram), fat percentage (fat / body-weight) and body water percentage (body-water / body-weight) of the three mice groups: LF, HF-OR and HF-OP (with 8, 5 and 5 individuals, respectively). When analyzing the individual differences in body content, we find that the fat percentage is positively correlated with the body-weight, and negatively correlated with the body-water percentage. When analyzing the group difference, figure 2 clearly shows that the HF-OP mice group, which is heaviest (the average 45 grams), has the lowest water percentage (the average 50 %) and the highest fat percentage (37 %). The average weight difference between the LF and HF-OR groups is not large (30.6 g and 33.8 g, respectively), but the fat-% and water-% are quite different. The lean contents for LF and HF-OR groups are similar (nearly 24 g), and the average lean content of the HF-OP group is only 2.5 grams higher than the other two groups.

[Fig 2. is here**]**

Indirect calorimetry

After taking the initial blood samples, the 20 mice were put into the indirect calorimetry box and the actual r_{CO2}, r_{O2} and RQ were measured over the two following days. The data around the interruptions (taking blood samples) were removed, and the IC data summary of the two days is listed in Table 4.

[Table 4 is here**]**

All observed values are equal within the error for day 1 and day 2, except for r_{CO2} of the LF mice. The r_{CO2} of the LF and HF-OR groups are similar and their difference is within the error, but the CO_2 production of the HF-OP group is about 8 ml/h more than that of the other two groups. The r_{O2} values differ significantly between the groups, and that of the HF-OP group is the highest.

The respiratory quotient (RQ) resembles the low-fat or high-fat food intake difference between the LF and the two HF groups. There is no difference between the HF-OR and HF-OP groups, and they are both lower than the RQ values of LF. As the Metabolic Rate values are directly computed from r_{CO2} and the RQ, they show the same trends. The MR value of HF-OP is highest (about 5 kJ/day more than HF-OR, 11 kJ/day more than LF).

Turnover rates k

The turnover rates for ^2H (k_{2H}), ^{18}O (k_{18O}) and ^{17}O (k_{17O}) were separately calculated from the logarithmic decline of the initial isotope abundance (2-hour after injection) and the isotope abundance of two finals (24h and 48h after the initial sample taken). The average k_{2H}, k_{18O}, k_{17O} for the three mice groups are shown in figure 3, whereas table 5 gives the numerical values, and in addition the turnover rate ratios and differences. All are presented for the 24hour and the 48hour period. It is clear that the LF group has the highest turnover rates of the three groups, and the differences are highly significant between LF and HF groups. The turnover rates of the HF-OR group are slightly higher than the HF-OP group, but for k_{2H} and k_{18O}, the difference is not significant.

[Fig 3. is here**]**

In table 5, it is clear that the uncertainty in k-48h is lower than that in k-24h, this is because of the larger difference between the isotope values for the 48h-finals and the initials. In table 5 and figure 3, for k_{2H}, we can see the difference between 24h- and 48h- is significant, k_2-48h being higher than k_{2H}-24h for all three mice groups. For k_{18O} and k_{17O}, on the other hand, the differences between the turnover rates for 24h- and 48h- are small and not significant. The k_{17O} and k_{18O} agree with each other within the uncertainties for both of the two times. This is to be

expected, as both the ^{17}O and ^{18}O label are subject to the same processes. The fact that they do agree within the uncertainty increases the confidence in the experimental results (both the animal handling side and the isotopic analysis of the blood samples). Therefore, it is possible to obtain the turnover of oxygen by taking the average of k_{17O} and k_{18O}, which lowers the uncertainty of k_O (turnover for oxygen isotopes).

As shown in table 5, the turnover rates ratios k_O/k_{2H} are typically 1.9 (LF) and 2.3 (HF) for 24h- finals, while for 48h-, they are a bit lower: 1.7 (LF), 2.0 (HF), fully caused by the increase of k_{2H}. In the analysis of k_O-k_{2H}, which carries the r_{CO2} signal information, the k_O-k_{2H} values are obviously lower for 48h- than for 24h- because of the higher k_{2H}-48h values. On the other hand, the differences between the three groups for the same final are not significant in most of the cases, expect the k_{17O} - k_{2H} for HF-OR group at 48h-final.

[Table 5 is here**]**

Total body water (TBW) and dilution space

The body dilution space (N) can be measured based on the plateau method (equation (6)) or on the intercept method (equation (7)), and the total body water is calculated using equation (9). In figure 4 we compare the body water percentage (water / mass) results calculated by TLW with those by EchoMRI. To illustrate the differences better, figure 4 shows the differences between the two. In terms of individual variation, the water percentage values from the calculation (TLW) and measurement (EchoMRI) are consistent with each other (not shown here). Moreover, the dilution space difference obtained by the intercept method based on the k-24h or k-48h is not significant (less than 0.1 %), so we only illustrate the percentage difference of the plateau and 48h-intercept method in figure 4.

[Fig 4. is here**]**

It is clear in figure 4, and expected (see above), that the plateau method gives higher water percentages than the intercept method for both ^{2}H, ^{18}O, and ^{17}O, and also that the water percentages based on ^{2}H are the highest (nearly 2 % higher than the EchoMRI values for the plateau method). On the other hand, the intercept method results for ^{18}O and ^{17}O give lower water percentages than that of EchoMRI. Still, given the combination of the indicated uncertainties for the TLW method (as indicated in figure 4) and the uncertainty of the EchoMRI method (estimated to be $\leq \pm$ 2 %), all differences shown in figure 4 are not significant. Furthermore, although the water percentage values for the three different mice groups are different (see figure 2), the differences between the TLW and EchoMRI methods do not seem to correlate with these percentages themselves.

[Table 6 is here**]**

The absolute total body water or dilution space (N) values calculated by three isotopes and two methods are listed in table 6 for the three mice groups. As figure 4 already showed, the values of the plateau method are higher than that of the intercept method for each mice group irrespective of the isotope used. N_{2H} is always higher than N_O, and N_{17O} matches N_{18O}. The most important feature in table 6 is that the dilution space of HF-OP group is significantly higher than that of LF and HF-OR, while the dilution spaces of HF-OR and LF are equal for most of the cases.

CO_2 production

The average r_{CO2} of each LF, HF-OR and HF-OP group are calculated by three kinds of equations which are listed in Table 3. We took the r_G=0.5. The r_{CO2} results are shown in figure 5, and for each group, there are two kinds of doubly labelled water methods: left columns based on ^{18}O and ^{2}H, and right columns based on ^{17}O and ^{2}H. We also consider the different dilution space (N) calculation methods (plateau or intercept methods) and two different finals (24h or 48h). The grey horizontal line in the bottom is the average r_{CO2} value for two days from the indirect calorimetry method for the three mice groups. Therefore, in summary, we consider 4 factors for each mice group to calculate the r_{CO2} : two finals (24h- or 48h-), two N calculation methods (plateau or intercept), three models (table 3, [4,45,46]), and two isotope combinations (based on ^{2}H-^{18}O or ^{2}H-^{17}O). The relative uncertainties of the classical DLW (^{18}O and ^{2}H) methods in this study are 8.5 % (24h-) and 5.1% (48h-), while for (^{17}O, ^{2}H) DLW they are 6.3 % (24h-) and 4.0 % (48h-). The relative uncertainty in the indirect calorimetry values is estimated to be $\leq \pm$ 2 %.

For all three mice groups, the 24h- r_{CO2} data are much higher than the 48h- data, irrespective of the calculation method, in other words, the differences between solid and hollow symbols in each column are the same. The differences are all caused by the k_{2H}-48h value being larger than the k_{2H}-24h one (see figure 3 and table 5). Of course, this difference could indicate a real different behavior, but the IC values for the two days do not show such a difference (table 4). In terms of different oxygen isotopes for each mice group, the difference is random (from 0 to 8 ml/h), just within the largest errors no matter which model is used. For each of the oxygen isotope and models in each mice group, the r_{CO2} calculated by the intercept method (yellow area) is lower than that by the plateau method. However, the differences are within the uncertainties. Interestingly, the intercept points are more scattered than the plateau ones, caused by the extra influence the turnover rates k have when using the intercept method. As was stated before, one can expect the plateau method to give an overestimation of the water pool size, and the intercept an underestimation. This results in and over- and underestimation of the r_{CO2}, respectively. The average of the two values would probably produce the best result, while their differences would give an estimate of the uncertainty.

[Fig 5. is here**]**

Obviously, the most striking feature of figure 5 is the discrepancy between all the TLW results on the one hand, and the IC results on the other. The discrepancy is the smallest for the 48h-two-pools models, with the solid triangles (Coward 1985, 48h-) having the lowest discrepancy with IC for each mice group. The lowest difference of TLW and IC is 8 ml/h (LF) and 16 and 15 ml/h (HF-OR and HF-OP) for these results (the equation 2-2 for 48h). The TLW and IC

results agree in the sense that both show the highest r_{CO2} for the HF-OP group. However, the average IC values for the HF-OR and LF groups are similar, while in the TLW methods, the r_{CO2} of HF-OR is higher than the r_{CO2} of the LF group (about 10 ml/h).

The discrepancy between TLW and IC needs an explanation. Figure 6a shows the individual r_{CO2} measured by the 2-1 and 2-2 (Coward 1985) models with the intercept method at 48h-finals, and the individual IC values of day two. It is very clear that r_{CO2} measured based on the ^{18}O-^{2}H and ^{17}O-^{2}H pairs are consistent, and their difference is within the uncertainty. For the LF and HF-OP groups, the r_{CO2} (^{18}O) is slightly higher than r'_{CO2} (^{17}O), but the difference is only about 4 ml/h and not significant. Figure 6b clearly illustrates the deviation of the TLW and IC methods. The r_{CO2} values of the TLW method are calculated by averaging the r_{CO2} (^{18}O) and r'_{CO2} (^{17}O), and the IC r_{CO2} values are the same as in figure 6a. The uncertainty in the difference (TLW minus IC) in figure 6b is around 3.5 ml/h (based on 3 % relative error for TLW and 2 % for IC). The average difference between the TLW and IC values for the LF mice is 7.2 ml/h, while the average distance between TLW and IC for HF groups is 18.1 ml/h, much larger than that of the LF group. The individual IC and TLW data show a similar pattern, which is a firm indication that the difference between TLW and IC is of a systematic nature. As the IC technique is straight forward and less assumption-prone, we suspect the deviation to be caused by isotopic effects not accounted for.

[Fig 6 a. is here**]**

[Fig 6 b. is here**]**

Discussion

The Triply Labelled Water method

Because the measurement of $\delta^{17}O$ has become simple, fast and accurate by the optical spectroscopic system, the classical Doubly Labelled Water (DLW) can easily be extended to Triply Labelled Water (TLW), and to our knowledge we demonstrated that here for the first time. The isotope abundance measurement uncertainties of ^{17}O and ^{18}O in the blood samples are similar, and the individual turnover rates of ^{17}O and ^{18}O are also expected to be equal no matter the subject treatment or the turnover time chosen, and our data confirmed this (figure 3). The same holds for the dilution space difference between N_{17O} and N_{18O} (figure 4). In terms of r_{CO2} calculated based on ^{18}O, ^{2}H, or on ^{17}O, ^{2}H, the values also match with each other for the same models (figure 5). These findings are consistent with our assumptions: although processes with ^{17}O and ^{18}O are governed by different fractionation factors, these differences can be accounted for, and do not cause a significant difference in the TLW method calculation. Moreover, using the TLW as extension to DLW, we can double-check the data quality of ^{18}O based on the ^{17}O data, and lower the measurement uncertainty of the CO_2 production. In this study, we lower the calculated r_{CO2} uncertainty to 3 % when taking the average of r_{CO2} (^{18}O, ^{2}H, 5 %) and r'_{CO2} (^{17}O, ^{2}H, 4 %).

Another use of the third isotope is to help quantify the evaporative water loss fraction, in other words, the TLW method enables us, at least in principle, to derive a direct estimate of the fractionated losses fraction (r_G). This value of fractional evaporative water losses - r_G – has been subject of discussion since many decades [4,5,48,49]. As the r_{CO2} (^{18}O, ^{2}H) should be equal to r'_{CO2} (^{17}O, ^{2}H), we can derive the individual r_G values by equating the two equations (11) and (12). However, the influence of the value of r_G is limited: in this paper, we took $r_G = 0.5$, a value that is also widely used in free-living mammals. If we would use $r_G = 0.25$ as other researchers have done [4,48], the r_{CO2} will increase by less than 2 %, which is within the uncertainty band of our TLW average values (3 %). Alternatively, one can say that in order to determine r_G from the combination (^{18}O, ^{2}H) and (^{17}O, ^{2}H) to ± 0.1, one would need an uncertainty in $r_{CO2} \leq \pm 1$ %, out of reach of the present measurement methods, as was already concluded in [5].

At the moment, highly enriched ^{17}O water is more expensive than pure ^{18}O water, this is mainly because of less demand for it. However, ^{17}O can now be easily detected by the optical systems such as the one we use. Also, one only needs a factor of 7 less ^{17}O label to achieve the same enrichment factor as for ^{18}O water, and this reduces the costs. Therefore, adding ^{17}O to the classical DLW method is practically easy now, and it is also worthy to use TLW to check the method and improve the precision of the CO_2 production.

Calculation protocols

We considered three models for CO_2 production, one is based on the single-pool model (1-1 and 1-2 in Table 3), another two series of equations are based on the two-pools model (Table 3). In addition, we used the best available values from the literature for the fractionation factors (including ^{17}O). The two-pool equations based on [46] take the individuals' specificity more into account, while the equations based on [45] use a group average for the dilution space ratio. We compared two different averaging methods for this R_{dil} in equation 3-1 and 3-2, one is for the whole group of 18 mice regardless of the feeding methods, the other is separate averages for the three mice subgroups (as used for figure 5). Differences in r_{CO2} were less than 2 %, so the group difference of R_{dil} is not essential. The results in figure 5 and 6 show that the r_{CO2} calculated by [46] are closer to the CO_2 production obtained by indirect calorimetry, but there still is a significant discrepancy, much larger for the 24h results than for the 48h ones, and much larger for HF groups than LF groups: The TLW method leads to higher numbers for the r_{CO2}.

We found a clear increase from k_{2H}-24h to k_{2H}-48h, but no significant change for k_{18O} and k_{17O}. This leads to increased values for r_{CO2} in the first 24 hours compared to the second 24 hours. One might speculate that this can be caused by the disturbance of the mice during the first day: we injected the labelled water, took the 2h- as well as the 24h- blood samples and put them in and out of the IC box at the first day, but we only took the 48h- samples on the second day. However, the IC results show no significant changes between the first and second day. Due to this low k_{2H}-24h, the TLW r_{CO2} results for the 24h deviate much more form the IC results than the 48h results (see figure 5). Still, also the 48h- results for r_{CO2} are high compared to the IC result, which fact one could alternatively formulate as: k_{2H}-24h is much too low, k_{2H}-48 is still too low, but by less. If we consider the IC results as straight forward and trustworthy, this would lead to the speculation that the ^{2}H label disappears form the body water at a lower rate than the water loss itself, so involving a process with very high fractionation. We discuss this possibility further below.

Influence of the nutritional conditions

Previous work in our lab focused on different nutrition and body composition effects on r_{CO2} of mice by the DLW technique [13]. They also separated their mice in the same three groups, only their mice were younger. They also found that the r_{CO2} measured by DLW matches IC results much better for low-fat mice than for the high-fat feeding mice, so the high-fat diet is a relevant factor to explain the overestimation of DLW. The main difference we consider between our three groups is the turnover rates difference (k_O-k_{2H}), because the TLW-determined body water agrees well with the EchoMRI-determined one, implying that the dilution space for the water is correctly determined. Yet, the r_{CO2} determined by TLW is systematically higher than that with IC, even for the "most agreeing" calculation method (see figure 5 and 6). This difference is the lowest, regardless of the individual difference, for the LF mice group, close to 8 ml/h, still significantly higher than the largest error of TLW (± 3 %) and IC (± 2 %). For the HF-OR and HF-OP mice groups, this difference is more than double that amount. The (too) high r_{CO2} values must be caused by too low k_{2H}-values, and/or too high k_{17O} and k_{18O} ones. The number of possible explanations is restricted, because the body water pool is correctly determined by TLW. The only thinkable way of getting too high ^{18}O and ^{17}O rates is assuming a strongly fractionating water loss process that preferably takes up ^{17}O and ^{18}O over ^{16}O, and with the same fractionation factor for both. Such a process is next to impossible to imagine, as fractionation factors for ^{17}O are typically half those for ^{18}O. For ^{2}H on the other hand, we would need an extra water loss or uptake process that heavily discriminates against ^{2}H, and such processes are thinkable, and in fact known: electrolysis of water, for example, manifests fractionations of -600 to -700 ‰ (so fractionation factors of 0.4 to 0.3), and also bacterial uptake is known to fractionate considerably (albeit not to the extent of electrolysis). A rough calculation shows that if a 10 % extra loss/exchange effect would exist with a ^{2}H fractionation factor of 0.4, this would lead to an overestimation of r_{CO2} by 10 ml/h. As the effect is considerably larger for the 24h than for the 48h results, the process might in fact be an exchange effect that reaches equilibrium at some point. If that is the case, the TLW-IC difference must gradually disappear. As the discrepancies are much larger for the HF than for the LF mice, body composition (fat content) and/or food digestion must play a profound role in this mechanism.

Conclusions

This study extends the traditional Double Labelled Water technique to Triply Labelled Water to estimate the CO_2 production for mice held in different nutrition conditions. Heavily enriched ^{17}O water is readily obtainable, and modern optical water isotope analysers, such as the OA-ICOS system used by us, produce ^{17}O values with the same ease as ^{18}O and ^{2}H results. At the moment, certified reference waters (such as [35]) are not yet available for highly enriched ^{17}O, so laboratories should make their own references by gravimetrical mixing. However, if demand increases, such reference waters will be made available, by international bodies such as IAEA, or commercial suppliers.

The results for both combinations ^{2}H-^{17}O and ^{2}H-^{18}O agree well, and hence the calculated r_{CO2} uncertainty is lower and the values are more robust. However, in this demonstration project, we were again confronted with systematic deviations between the DLW (now TLW) method

and indirect calorimetry as earlier found in [13]. An extra advantage of using both ^{18}O and ^{17}O is that the overestimation of the CO_2 production by TLW cannot be due to some extra process influencing the oxygen side through fractionation, as then ^{17}O and ^{18}O would be influenced differently, and their results would differ. Therefore, we can conclude that a process on the hydrogen side must be the culprit: some extra water removal / uptake with a high degree of discrimination against ^{2}H can explain the too high r_{CO2} results. This uptake apparently is dependent on the food intake and / or the body composition. More detailed isotope analysis (such as gastric fluids [50]) can probably reveal this extra water loss / exchange channel.

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Conflicts of interest/Competing interests (include appropriate disclosures) None of the authors reported a conflict of interest.

Availability of data and material (data transparency) All data described in the manuscript are in the database system of the Centre for Isotope Research, and are available upon request.

Authors' contributions HAJM initiated the research. All authors contributed to the study concept and design. Material preparation and all experiments on and handing of the mice were conducted by DK and GvD. Micro-distillation and isotopic measurement of the blood samples were done by XW. Data collection and analysis were performed by XW and HAJM. HAJM wrote the data processing code. The draft of the manuscript was written by XW. All authors read, commented and approved the final manuscript.

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Table 1. Isotope δ-values of the enriched TLW reference waters. The δ^2H and $\delta^{18}O$ values were measured by OA-ICOS and calibrated using the IAEA-reference waters. The $\delta^{17}O$ values are based on several dilution experiments, with a relative uncertainty of ± 1 % attributed to them.

Enriched TLW reference water	δ^2H (‰)	Measured error (‰)	$\delta^{18}O$ (‰)	Measured Error (‰)	$\delta^{17}O$ (‰)	Fitted error (‰)
REF-1	13714	12	2009.2	2.3	1630	16
REF-2	11079	9	1619.9	2.3	1300	13
REF-3	8400	9	1225.2	2.2	1000	10
REF-4	4370	8	636.0	1.0	520	5

Table 2. Isotope fractionation factors measured in vitro for the equilibrium and kinetic exchanges of ^2H, ^{18}O and ^{17}O between liquid water and vapor, and for the ^{18}O, ^{17}O between water and gaseous CO_2 at 37°C [4,15,17–19,25].

Fractionation factor	equilibrium	kinetic	Final factors used in this study from equilibrium: kinetic=3:1
f_1	0.941	0.9235	0.9366
$f_{2,18O}$	0.9925	0.976	0.9884
$f_{3,18O}$	1.0389		
$f_{2,17O}$	0.996	0.9872	0.9938
$f_{3,17O}$	1.0202		
$(f_{2,18O} - f_1)/2f_{3,18O}$			0.0249
$(f_{2,17O} - f_1)/2f_{3,17O}$			0.0280

Table 3. CO_2 production (r_{CO2}) calculation equations by the doubly labelled water method separately based on ^{18}O or ^{17}O with ^2H [4,45,46].

No.	Author	pools	Equation	N
1-1	Speakman Book 1997	1	$r_{CO2} = \frac{N}{2.0778}(k_{18O} - k_{2H}) - r_G \times 0.0249 N k_{2H}$	$N = N_{18O}$
1-2			$r'_{CO2} = \frac{N}{2.0404}(k_{17O} - k_{2H}) - r_G \times 0.028 N k_{2H}$	$N = N_{17O}$
2-1	Coward 1985	2	$r_{CO2} = \frac{1}{2.0778}(N_{18O}k_{18} - N_{2H}k_{2H}) - r_G \times 0.0249 N_{2H}k_{2H}$	
2-2			$r'_{CO2} = \frac{1}{2.0404}(N_{17O}k_{17O} - N_{2H}k_{2H}) - r_G \times 0.028 N_{2H}k_{2H}$	
3-1	Speakman 1993	2	$r_{CO2} = \frac{N}{2.0778}(k_{18O} - R_{dil}k_{2H}) - r_G \times 0.0249 N R_{dil}k_{2H}$	$N = \frac{\left(N_{18O} + \frac{N_{2H}}{R_{dil}}\right)}{2}$
3-2			$r'_{CO2} = \frac{N}{2.0404}(k_{17O} - k_{2H}) - r_G \times 0.028 N R_{dil}k_{2H}$	$N = \frac{\left(N_{17O} + \frac{N_{2H}}{R_{dil}}\right)}{2}$

Table 4. Indirect calorimetry (IC) measurement results for two days, expressed as mean ± standard error (SE). Day 1 means the first 24 hours in the IC box after the initial samples taken, and Day 2 is the second 24 hours. rCO_2 and rO_2 are the CO_2 production and O_2 consumption in ml per hour, RQ is the respiratory quotient. The metabolic rates (MR) are calculated based on [38].

	time	LF (n=8)	HF-OR (n=5)	HF-OP (n=5)
rCO_2 (ml/h)	Day 1	77.8 ± 2.1	83 ± 4	91.4 ± 2.0
	Day 2	83.4 ± 1.4	83.0 ± 2.4	91 ± 3
rO_2 (ml/h)	Day 1	99.1 ± 2.3	118 ± 5	128 ± 4
	Day 2	100.5 ± 1.9	113 ± 4	124 ± 3
RQ (CO_2/O_2)	Day 1	0.785 ± 0.017	0.705 ± 0.008	0.713 ± 0.009
	Day 2	0.828 ± 0.006	0.736 ± 0.008	0.730 ± 0.008
MR (kJ/day)	Day 1	47.5 ± 0.9	55.0 ± 2.2	59.8 ± 1.6
	Day 2	48.4 ± 0.7	53.0 ± 1.7	58.3 ± 1.6

Table 5. The turnover rates ($10^{-3}h^{-1}$) for ^2H (k_{2H}), ^{18}O (k_{18O}) ^{17}O (k_{17O}); turnover rates ratio: k_{18O}/k_{2H} and k_{17O}/k_{2H}; turnover rates differences ($10^{-3}h^{-1}$): $k_{18O} - k_{2H}$ and $k_{17O} - k_{2H}$. The ratios and differences are calculated from the individual initial isotope abundances (2-hour after injection) and two finals (24h and 48h after the initial sample taken), and are expressed as mean ± SE.

24-hour-final							
Mice group	k_{2H} ($10^{-3}h^{-1}$)	k_{18O} ($10^{-3}h^{-1}$)	k_{17O} ($10^{-3}h^{-1}$)	k_{18O} / k_{2H}	k_{17O} / k_{2H}	$k_{18O} - k_{2H}$ ($10^{-3}h^{-1}$)	$k_{17O} - k_{2H}$ ($10^{-3}h^{-1}$)
LF (n=8)	10.2 ± 0.7	19.3 ± 0.8	19.5 ± 0.8	1.93 ± 0.07	1.94 ± 0.07	9.13 ± 0.19	9.3 ± 0.3
HF-OR (n=5)	7.4 ± 0.7	17.0 ± 1.0	17.1 ± 1.1	2.33 ± 0.12	2.34 ± 0.11	9.6 ± 0.4	9.6 ± 0.5
HF-OP (n=5)	7.4 ± 0.5	16.8 ± 0.4	16.4 ± 0.4	2.28 ± 0.08	2.23 ± 0.10	9.38 ± 0.16	9.0 ± 0.3
48-hour-final							
Mice group	k_{2H} ($10^{-3}h^{-1}$)	k_{18O} ($10^{-3}h^{-1}$)	k_{17O} ($10^{-3}h^{-1}$)	k_{18O} / k_{2H}	k_{17O} / k_{2H}	$k_{18O} - k_{2H}$ ($10^{-3}h^{-1}$)	$k_{17O} - k_{2H}$ ($10^{-3}h^{-1}$)
LF (n=8)	11.6 ± 0.4	19.7 ± 0.4	19.7 ± 0.5	1.710 ± 0.018	1.71 ± 0.021	8.16 ± 0.13	8.15 ± 0.17
HF-OR (n=5)	8.9 ± 0.3	17.5 ± 0.4	17.7 ± 0.4	1.97 ± 0.05	2.0 ± 0.04	8.6 ± 0.3	8.79 ± 0.23
HF-OP (n=5)	8.54 ± 0.21	16.9 ± 0.3	16.5 ± 0.4	1.98 ± 0.03	1.9 ± 0.03	8.37 ± 0.16	7.97 ± 0.22

Table 6. The dilution space calculated using either ^2H (N_{2H}), ^{18}O (N_{18O}) and ^{17}O (N_{17O}) with the plateau or intercept methods for the three mice groups. The values are expressed as mean ± SE.

Mice group	N_{2H} (mol)		N_{18O} (mol)		N_{17O} (mol)	
	Plateau	Intercept	Plateau	Intercept	Plateau	Intercept
LF (n=8)	1.17 ± 0.03	1.14 ± 0.03	1.156 ± 0.025	1.111 ± 0.025	1.134 ± 0.022	1.091 ± 0.022
HF-OR (n=5)	1.153 ± 0.022	1.13 ± 0.03	1.142 ± 0.021	1.102 ± 0.021	1.122 ± 0.023	1.085 ± 0.023
HF-OP (n=5)	1.30 ± 0.04	1.27 ± 0.04	1.29 ± 0.04	1.24 ± 0.04	1.28 ± 0.04	1.24 ± 0.04

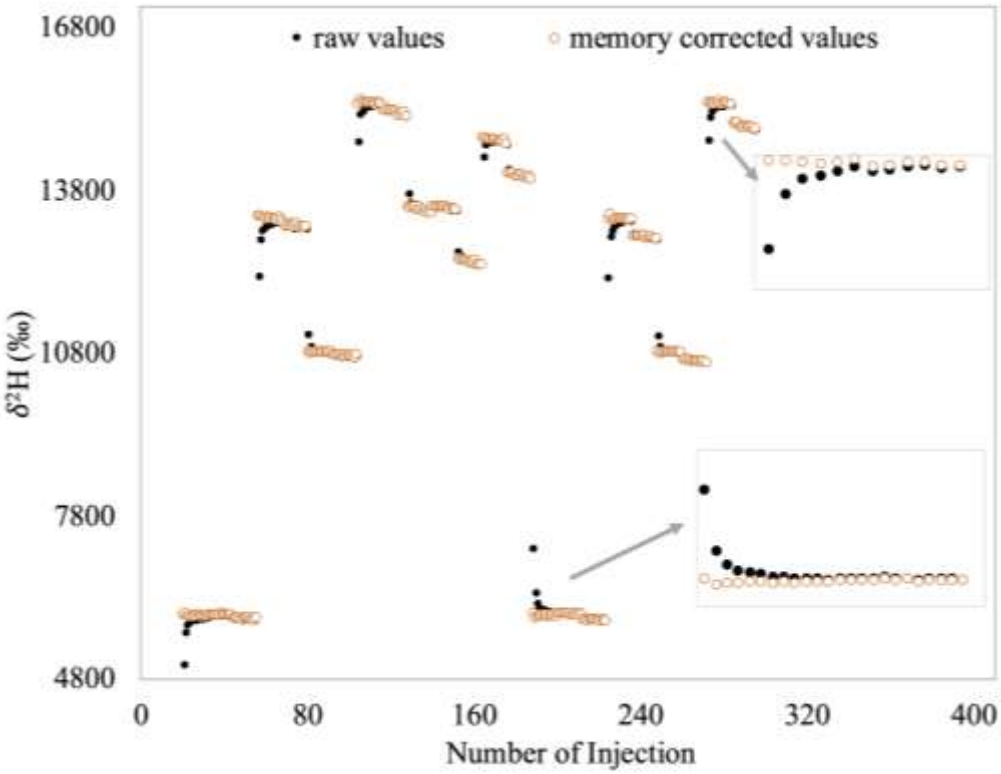

Figure 1. The memory effect of δ^2H observed in an initial samples' measurement batch. The measured (raw) values are showed by the black circles and the open red circles indicate the values after memory effect correction by the 3 pools exchange algorithm. In addition to the memory, there is also some drift visible which is also corrected by the data analysis program.

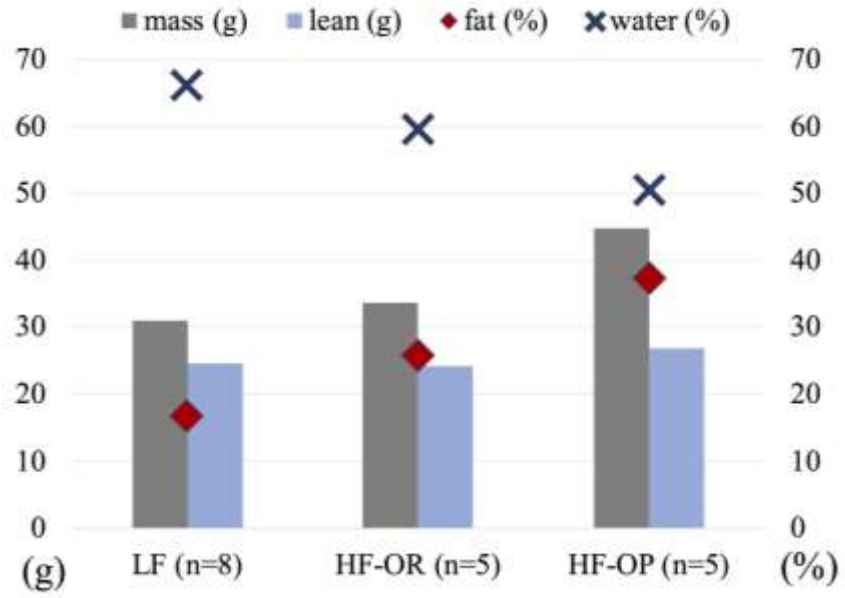

Figure 2. The average body-weight (g), fat percentage, body-water percentage and lean content (g) for the low fat (LF) mice group, high fat obesity-resistant (HF-OR) mice group and high fat obesity-prone (HF-OP) mice group.

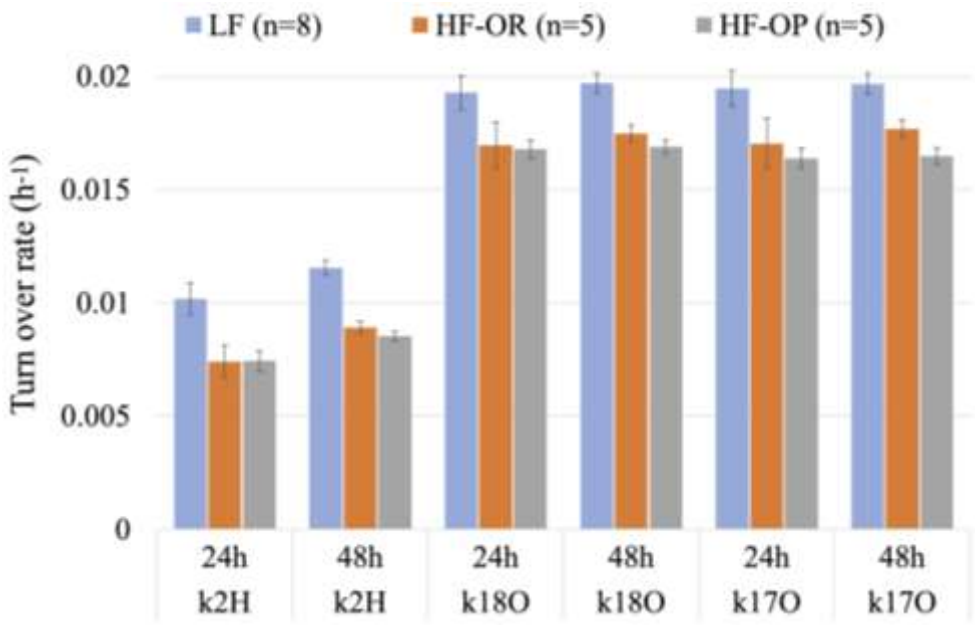

Figure 3. The average turnover rates (h^{-1}) of ^{2}H (k_{2H}), ^{18}O (k_{18O}) and ^{17}O(k_{17O}) for the LF (blue, n=8), HF-OR (red, n=5), and HF-OP (grey, n=5) mice groups based on the final samples taken at 24hour or 48hour after taking the initials. The error bar is ± SE.

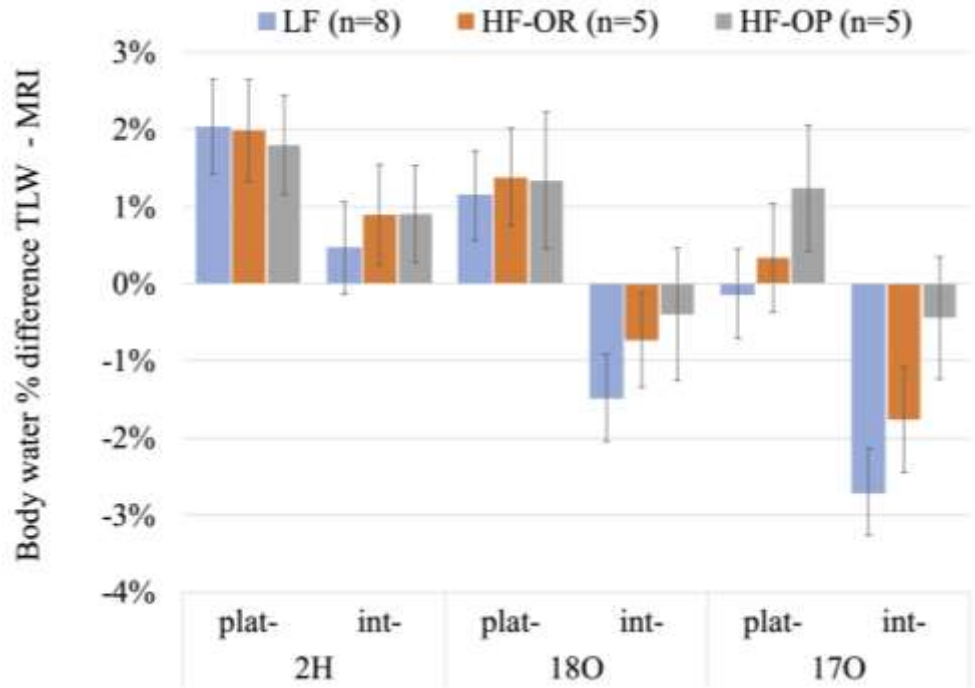

Figure 4. Body water percentage differences between TLW (both ^{2}H, ^{18}O, and ^{17}O) and EchoMRI for the three mice groups, expressed as mean ± SE. Plat- means using the plateau method (equation (6)), an int- means using the intercept method (equation (7)). The uncertainty for the EchoMRI values is estimated to be 2%.

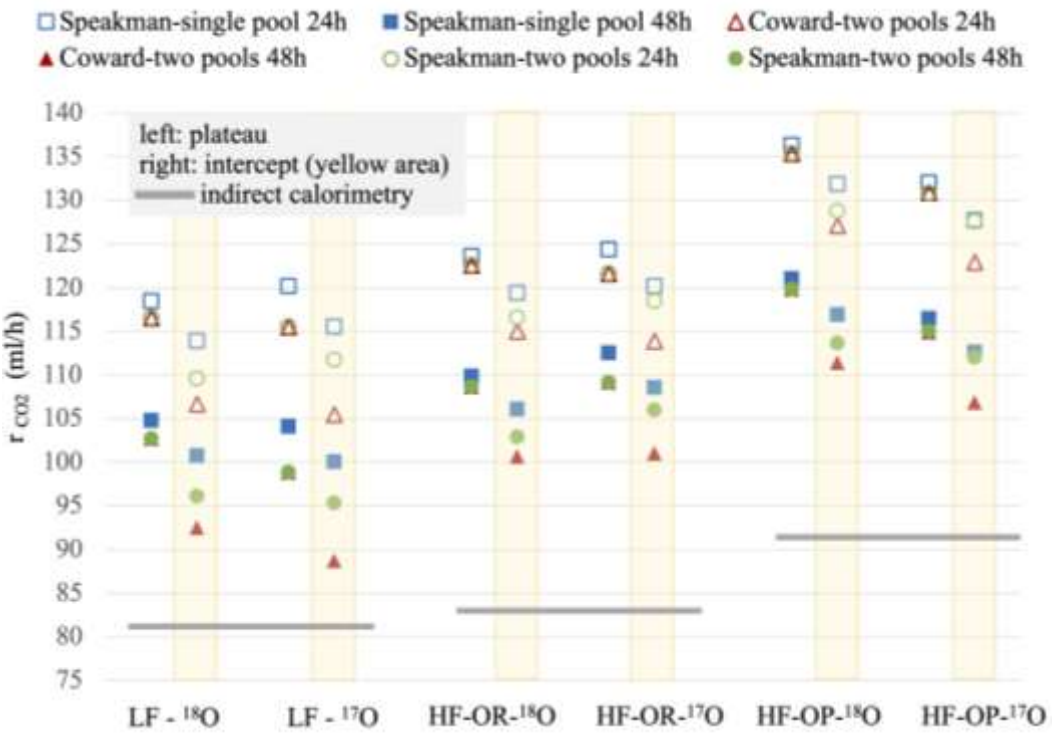

Figure 5. The average r_{CO2} (ml / h) of the three mice groups (LF, HF-OR and HF-OP) based on different calculation protocols. There are three models (Speakman single- or two-pools model, Coward two-pools model), two kinds of doubly labelled water method (^{18}O - ^{2}H, or ^{17}O - ^{2}H), two dilution space calculation methods (plateau or intercept), and two different finals (24h or 48h). The grey horizontal lines in the bottom are the average r_{CO2} values of two days from the indirect calorimetry method.

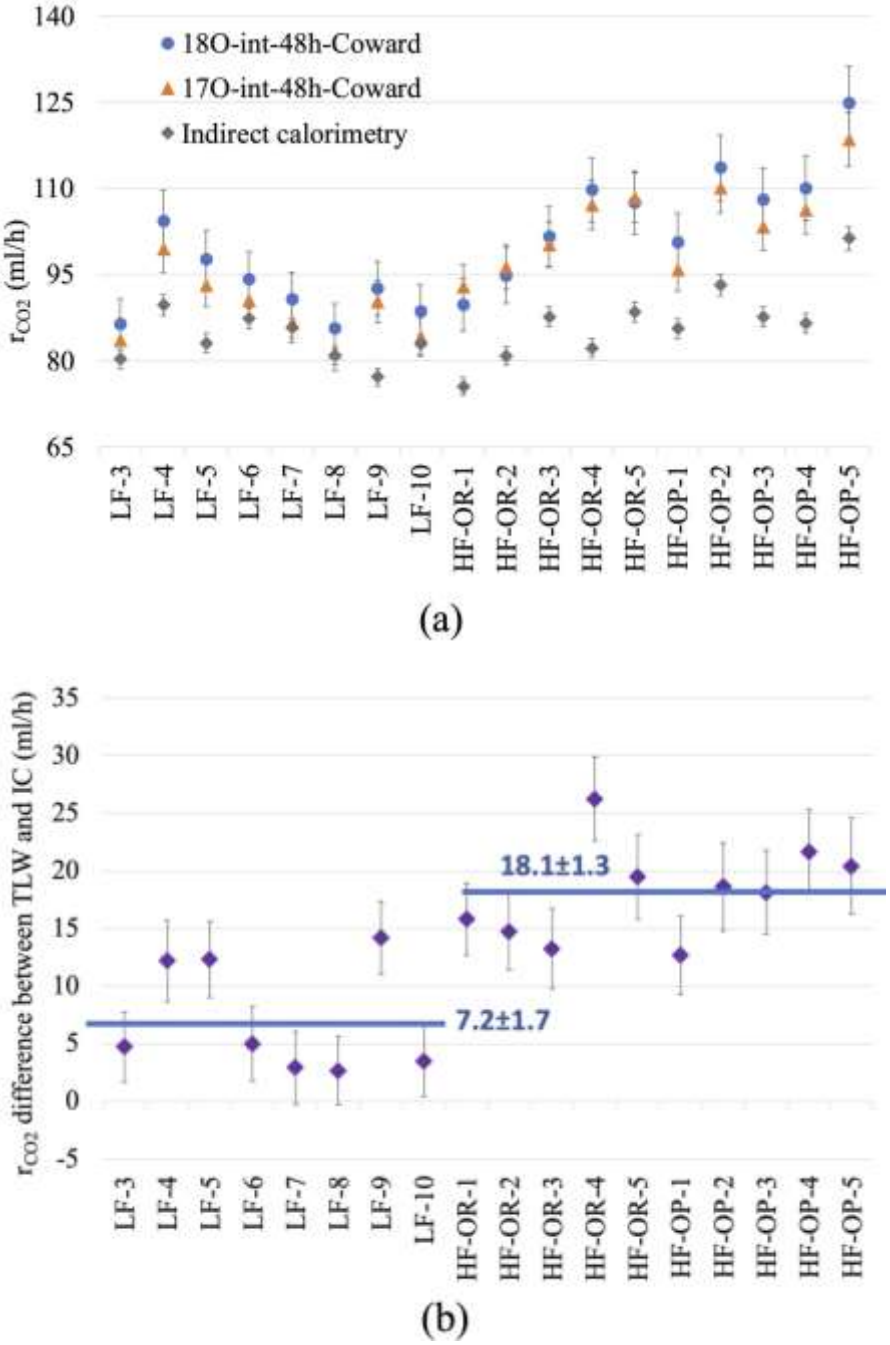

Figure 6 (a) Individual r_{CO2} values (ml/h) measured by ^{18}O-^{2}H or ^{17}O-^{2}H DLW using the Coward 1985 two-pools model by the intercept method at the 48h finals. The blue points are the ^{18}O-based results, with 5.1% uncertainty, and the orange points are for ^{17}O with 4% uncertainty. The grey points are the individual indirect calorimetry vales for day two (2% uncertainty).
(b) The difference between the r_{CO2} values calculated by the TLW and IC methods. The r_{CO2} values of the TLW method are the average the r_{CO2} (^{18}O) and r'_{CO2} (^{17}O) in figure 6 A. The uncertainty in the difference is around 3.5 ml/h. The values in the plot are the mean ± the SE, for LF, and HF (OR and OP taken together)