Potential Bias of Doubly Labeled Water for Measuring Energy Expenditure Differences Between Diets Varying in Carbohydrate

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Abstract

Objective: To examine possible bias of the doubly labeled water (DLW) method for measuring energy expenditure (EE_{DLW}) in humans consuming a low-carbohydrate diet.

Methods: EE_{DLW} was measured during the final two weeks of a month-long baseline diet (BD; 50% carbohydrate, 35% fat, 15% protein) as well as a subsequent isocaloric ketogenic diet (KD; 5% carbohydrate, 80% fat, 15% protein) in 17 men with BMI between 25-35 kg/m^2. Physical activity was measured by accelerometers. Subjects resided two days per week in respiratory chambers to measure energy expenditure ($EE_{chamber}$). Body composition and energy intake measurements were used to calculate expenditure by energy balance (EE_{bal}).

Results: Neither $EE_{chamber}$ nor EE_{bal} were significantly different during the KD versus the BD phase ($\Delta EE_{chamber}$=24±30 kcal/d; p=0.43 and ΔEE_{bal}=-141±118 kcal/d; p=0.25). Similarly, physical activity (-5.1±4.8%; p=0.3) and the exercise efficiency (-1.6±2.4%; p=0.52) were unchanged. However, EE_{DLW} was 209±83 kcal/d higher during the KD versus the BD (p=0.023).

Conclusions: The increased EE_{DLW} during the KD was incommensurate with the respiratory chamber, energy intake and body composition measurements and could not be explained by objective measures of physical activity or exercise efficiency. Our data raise the possibility of systematic bias of the DLW method for low-carbohydrate diets.

Keywords: Energy expenditure; doubly labeled water, diet composition

What is already known about this subject?

- The doubly labeled water (DLW) method has been successfully applied to measuring energy expenditure in humans since the 1980s and has been validated in comparison to respiratory chamber measurements in subjects consuming moderate carbohydrate diets.
- The DLW has never been validated in humans consuming a very low-carbohydrate diet and there are theoretical reasons why the DLW method might result in a systematic bias when calculating energy expenditure differences between diets widely varying in carbohydrate.

What this study adds:

- While not designed as a validation experiment, this two-month long inpatient isocaloric feeding study represents the most rigorously controlled investigation of the DLW method during a low-carbohydrate diet in humans.
- The DLW method calculated a significant increase in energy expenditure after transitioning from a moderate carbohydrate diet to an isocaloric very low carbohydrate, ketogenic diet. However, the increased expenditure measured by the DLW method was inconsistent with both the coincident respiratory chamber measurements, changes in body energy stores, physical activity, and exercise efficiency measurements.
- Our data raise the possibility that the DLW method results in a systematic bias when calculating energy expenditure differences between diets varying widely in the proportion of carbohydrate.

Introduction

The doubly labeled water (DLW) method was devised in 1955 by Lifeson et al. for measuring daily energy expenditure averaged over periods of several days (1). While originally applied to small animals, the method has provided important insights regarding human energy metabolism since the 1980s (2, 3, 4, 5). More recently, the DLW method was used in a controlled feeding study in humans to conclude that low-carbohydrate diets result in substantially increased total energy expenditure compared to isocaloric diets with higher proportions of carbohydrate (6). However, such results appear to run counter to several controlled feeding studies that employed respiratory chambers to measure energy expenditure that found no such expenditure increases with isocaloric lower carbohydrate diets (7). Forthcoming studies rely on the DLW method for assessing the energy expenditure effects of diets varying widely in the proportion of dietary carbohydrate (8), but the DLW has never been validated in humans consuming low-carbohydrate diets.

There are theoretical reasons why the DLW method might result in a systematic bias when calculating energy expenditure differences between diets widely varying in carbohydrate (9, 10, 11). Specifically, whereas the DLW method assumes that deuterium is lost only via body water, deuterium is also incorporated into other molecules (lipids, protein, glycogen, DNA, etc.) via processes at rates likely dependent on dietary carbohydrate and circulating hormones such as insulin. For example, the Supplementary Materials provides a calculation of the systematic bias in energy expenditure introduced by possible variations in the de novo lipogenesis pathway in humans consuming diets varying widely in carbohydrate content.

Here, we explore the potential bias of the DLW method using data from our previously published study (12) in which 17 men were transitioned from a one-month inpatient run-in period consuming a moderate-carbohydrate baseline diet (BD) (50% carbohydrate, 35% fat, 15% protein) directly to a second month-long inpatient period consuming an isocaloric ketogenic diet (KD) (5% carbohydrate, 80% fat, 15% protein).

Methods

Details of the study and methods were reported previously (12). We studied 17 men with BMI between 25-35 kg/m^2 who were admitted as inpatients to metabolic wards where they consumed a standard baseline diet (BD) composed of 50% energy from carbohydrate, 35% fat, and 15% protein for 4 weeks immediately followed by 4 weeks of an isocaloric very low-carbohydrate, ketogenic diet (KD) composed of 5% carbohydrate, 80% fat, 15% protein. Body weight and height were measured to the nearest 0.1 kg and 0.1 cm, respectively, with subjects wearing a hospital gown and undergarments and following an overnight fast. Body fat was measured using DXA scanners (Lunar iDXA, GE Healthcare, Madison, WI, USA).

Subjects spent two consecutive days each week residing in respiratory chambers to measure energy expenditure ($EE_{chamber}$). As described previously (12), during the BD period, the daily energy expenditure was calculated as follows:

$$EE_{chamber}\,(\text{kcal}) = 3.88 \times VO_2\,(\text{L}) + 1.08 \times VCO_2\,(\text{L}) - 1.57 \times N\,(\text{g})$$

where VO_2 and VCO_2 were the volumes of oxygen consumed and carbon dioxide produced, respectively, and N was the 24hr urinary nitrogen excretion measured by chemiluminescence

(Antek MultiTek Analyzer, PAC, Houston, TX). During the KD period, the equations were adjusted to account for 24-hour urinary ketone excretion, K_{excr}:

$$EE_{chamber}\,(\text{kcal}) = 3.88 \times \left[VO_2\,(\text{L}) - 0.32\ (\text{L/g}) \times K_{excr}\,(\text{g})\right] + 1.08 \times VCO_2\,(\text{L}) - 1.57 \times N(\text{g}) + 1.39 \times K_{excr}\,(\text{g})$$

Energy efficiency of physical activity was measured in the respiratory chamber with subjects exercising at a constant, self-selected, level of moderate-intensity cycle ergometry.

Energy expenditure was measured by DLW (EE_{DLW}) during the final two weeks of the BD and KD periods to allow sufficient time for fluid shifts as subjects to adjusted to each diet. Subjects drank from a stock solution of ^2H_2O and $H_2{}^{18}O$ water in which 1 g of ^2H_2O (99.99% enrichment) was mixed with 19 g of $H_2{}^{18}O$ (10% enrichment). An aliquot of the stock solution was saved for dilution to be analyzed along with each set of urine samples. The water was weighed to the nearest 0.1 g into the dosing container. The prescribed dose was 1.0 g per kg body weight and the actual dose amounts were entered in the dose log. Spot urine samples were collected daily. Isotopic enrichments of urine samples were measured by isotope ratio mass spectrometry. The average CO_2 production rate (rCO_2), corrected for previously administered isotope doses, can be estimated from the rate constants describing the exponential disappearances of the labeled ^{18}O and deuterated water isotopes (k_O and k_D) in repeated spot urine samples collected over several days. We used the parameters of Racette et al. (13) with the weighted dilution space calculation, R_{dil}, proposed by Speakman (14):

$$\begin{aligned} rCO_2 &= \left(N/2.078\right)\left(1.007k_O - 1.007R_{dil}k_D\right) - 0.0246r_{GF} \\ r_{GF} &= 1.05\left(1.007k_O - 1.007R_{dil}k_D\right) \\ R_{dil} &= \left[\left(N_D/N_O\right)_{ave} \times n + 1.034 \times 255\right] \Big/ \left(n + 255\right) \end{aligned}$$

where r_{GF} accounts for the fractionation of the isotopes and $(N_D / N_O)_{ave}$ is the mean of the N_D / N_O values from the n=17 subjects.

During the baseline period, EE_{DLW} was calculated as:

$$EE_{DLW}\,(\text{kcal}) = \left[\frac{3.85}{RQ} + 1.07\right] \times rCO_2\,(\text{L})$$

where the respiratory quotient, RQ, was calculated as the average 24hr RQ measured during the metabolic chamber days. During the KD period, EE_{DLW} was calculated as:

$$EE_{DLW}\,(\text{kcal}) = \left[\frac{3.85}{RQ} + 1.07\right] \times rCO_2\,(\text{L}) - \left[3.85 \times 0.32 + 1.39\right] \times K_{excr}\,(\text{g})$$

Energy expenditure as calculated by energy balance (EE_{bal}) was determined using the daily metabolizable energy intake (EI) along with the measured rates of change of the body energy storage pools determined from body composition measurements at the beginning and end of each two-week BD and KD period coincident with the DLW measurements, dFM/dt and dFFM/dt, according to the following equation:

$$EE_{bal}\,(\text{kcal/d}) = EI\,(\text{kcal/d}) - 1100(\text{kcal/kg}) \times \frac{dFFM}{dt} - 9300(\text{kcal/kg}) \times \frac{dFM}{dt}$$

As opposed to our previous study (12) that reported results during the entire six-week period when EI was held constant (i.e. the last two weeks of the BD phase and the entirety of the KD phase), we now report results based upon data obtained only during the two-week DLW phase of both BD and KD periods. In addition to allowing direct comparison with the coincident DLW measures, focusing on the last two weeks of each diet allowed for dissipation of any transient

changes in energy expenditure needed to adapt to new fuel sources, whether transitioning from the usual diet to the BD or the change from BD to the KD.

Statistical analysis was performed using a paired, two-sided t-test with significance declared at the $p<0.05$ threshold. The data are reported as mean±SE.

Results

During the final two weeks of the BD, EI was 2738±107 kcal/d and EE_{DLW} was 2964±126 kcal/d, indicating that the subjects were in a state of negative energy balance (-227±79 kcal/d; p=0.011) as shown in Figure 1A. Accordingly, subjects lost weight and body fat during the BD phase. The calculated energy expenditure – taking into account the changes in body energy stores – was EE_{bal} = 3136±171 kcal/d and was not significantly different from EE_{DLW} (p=0.23). However, EE_{DLW} was 338±77 kcal/d higher (p=0.0005) than $EE_{chamber}$ = 2626±104 kcal/d, indicating that subjects expended significantly more energy outside the chamber during the BD phase.

During the final two weeks of the KD phase, EI was 2730±110 kcal/d which (by design) was not significantly different from the BD phase (p=0.16). Whereas we previously reported a transient increase in $EE_{chamber}$ during the first two weeks after introducing the KD (12), neither $EE_{chamber}$ (2650±89 kcal/d; p=0.43) nor EE_{bal} (2995±160 kcal/d; p=0.25) were significantly different during the last two weeks of the KD compared to the last two weeks of the BD coincident with the DLW measurement periods. Likewise, physical activity measured using an accelerometer mounted on the hip was not significantly different (KD relative to BD, -5.1±4.8%; p=0.3); and

energy efficiency of physical activity measured in the respiratory chamber with subjects exercising at a constant level of moderate-intensity cycle ergometry was not significantly different (-1.6±2.4%; p=0.52) between the BD and KD phases.

Despite no significant differences in EI, $EE_{chamber}$, EE_{bal}, physical activity, or exercise efficiency between the KD and BD phases (Figure 1B), EE_{DLW} was 209±83 kcal/d higher during the KD phase (p=0.023). The changes in EE_{DLW} and EE_{bal} were in opposite directions indicating that the increase in EE_{DLW} during the KD was incommensurate with the changes in body weight and fat mass.

During the BD, $EE_{chamber}$ was highly correlated with EE_{bal} (Figure 2A, r=0.85; p<0.0001), as were the correlations between $EE_{chamber}$ and EE_{DLW} (Figure 2B, r=0.79; p=0.0002) and between EE_{DLW} and EE_{bal} (Figure 2C, r=0.62; p=0.008). The correlation between $EE_{chamber}$ and EE_{bal} remained high during the KD (Figure 2D, r=0.88; p<0.0001), whereas the correlation between $EE_{chamber}$ and EE_{DLW} was somewhat attenuated (Figure 2E, r=0.58, p=0.01), and EE_{DLW} was not significantly correlated with EE_{bal} (Figure 2F, r=0.40; p=0.1). In other words, whereas EE_{DLW} was highly correlated with both $EE_{chamber}$ and EE_{bal} during the BD, and $EE_{chamber}$ remained highly correleated with EE_{bal} during the KD, the correlations with EE_{DLW} were diminished.

There were two clear EE_{DLW} outliers. The first outlier, “subject A”, had an EE_{DLW} that was 1220 kcal/d greater than EI during the BD, and was 1751 kcal/d greater than EI during the KD despite having slight gains in weight and body fat during these periods. In contrast, the $EE_{chamber}$ measurements for this subject were only 173 kcal/d less than EI during the BD and 65 kcal/d less

than EI during the KD. The second outlier, "subject B", had an EE_{DLW} during the BD that was only 123 kcal/d higher than $EE_{chamber}$, but during the KD his EE_{DLW} increased by 1136 kcal/d which was ~3 standard deviations greater than the mean increase in $EE_{DLW,}$ suggesting severe negative energy balance despite the subject gaining weight during this period and $EE_{chamber}$ increasing by only 72 kcal/d. Supplementary Tables S1-S4 provide data on the energy expenditure comparisons between BD and KD phases with and without the exclusion of these subjects. The increase in EE_{DLW} was 126±62 kcal/d after excluding these subjects was not significant (Supplementary Table 4; p=0.062).

Discussion

Our inpatient isocaloric feeding study represents the most rigorously controlled investigation of the DLW method during low-carbohydrate diets in humans. Four days of respiratory chamber measurements that were coincident with each DLW period did not detect significant changes in energy expenditure. The observed increase in EE_{DLW} after transitioning to the KD was substantially greater than could be accounted for by changes in body energy stores. Whereas individual measurements of $EE_{chamber}$ and EE_{bal} were highly correlated with each other during both diet periods, EE_{DLW} was only highly correlated with these measurements during the BD. Thus, the KD period appeared to result in DLW data that were inconsistent with the other expenditure measurements.

We previously reported that $EE_{chamber}$ transiently increased during the first two weeks of the KD but there was no significant difference by the end of the KD (12). The current study shows that $EE_{chamber}$ during the final two weeks of the KD was not significantly different from the final two

weeks of the BD. These periods were coincident with the DLW measurements that indicated an increase in energy expenditure. The discrepancy between the respiratory chamber and DLW measurements was previously explained by increased physical activity outside the respiratory chamber that might have been responsible for the increased expenditure detected by the DLW method during the KD (12). However, this potential explanation does not agree with the lack of increase in objectively measured physical activity. Also, the energy expended to perform the same low-intensity exercise in the respiratory chamber was not significantly changed by the KD, making it unlikely that the energy efficiency of skeletal muscle contraction had been altered after transitioning to the KD. Thus, the DLW data were discordant with several independent measures all indicating that the KD did not result in significant energy expenditure changes.

Whereas respiratory chamber measurements have high-precision, with an intrasubject coefficient of variation of $EE_{chamber}$ ~2-3% (15), the DLW method is less precise with an intrasubject coefficient of variation of EE_{DLW} ~ 8-15% (16). Thus, the relatively large inherent variability of the DLW method may have led to an apparent increase in EE_{DLW} during the KD simply by chance (type-1 error). And, we cannot definitively exclude the possibility of a real increase in energy expenditure, especially at the modest effect size of ~126 kcal/d after excluding two likely DLW outliers. Nevertheless, our results raise the possibility that the measured increase in EE_{DLW} after transitioning to a very low-carbohydrate diet may have been due to a methodological bias.

The apparent simplicity of the DLW method, along with the elegance of the underlying theory, belies the fact that its practical application requires many assumptions. Various amendments to the original equations have been devised to account for deviations from the original assumptions

of the DLW method, and these revised equations introduce additional parameters and assumptions. Depending on the choice of assumptions and corresponding parameters, the calculated average rate of CO_2 production can vary by as much as 15% (17). Furthermore, converting the rate of CO_2 production to energy expenditure requires assumptions about the energy equivalent of CO_2 that varies substantially depending on the diet composition and the state of energy balance.

To inform the choice of equations, calibration and validation studies have been performed in humans where the mean CO_2 production rate measured using the DLW method has been compared to simultaneous respiratory chamber measurements; the DLW energy expenditure estimates have also been compared with weight-maintaining metabolizable energy intake of typical western diets with moderate proportions of carbohydrate (i.e., ~50% of energy) (4). However, the DLW method has never been validated in humans consuming a low-carbohydrate diet.

Estimates of energy expenditure based on the DLW method can be influenced by the fluxes through biosynthetic pathways that likely vary substantially depending on the carbohydrate content of the diet, especially the de novo lipogenesis pathway (9, 10, 11). However, the magnitude of this potential bias in humans was thought to be relatively small, amounting to a difference in EE_{DLW} by only about 30-60 kcal/d (see Supplementary Materials). Interestingly, even this small systematic bias would have been sufficient to nullify the statistical significance of the observed increased EE_{DLW} in our study. Furthermore, we cannot rule out the possibility of a larger EE_{DLW} bias with a low carbohydrate diet without direct measurements of changes in de

novo lipogenesis or other contributing metabolic fluxes. Individual variability of de novo lipogenesis changes after transitioning to the KD might explain the diminished correlations during the KD between EE_{DLW} with $EE_{chamber}$ and EE_{bal}.

In support of the concept that EE_{DLW} may overestimate energy expenditure differences between diets varying widely in carbohydrate content, a recent study in mice found that the DLW method overestimated CO_2 production during low-carbohydrate, high-fat diets, but agreed with respiratory chamber measurements during a high-carbohydrate diet (18). However, such results have been questioned based on criticisms of the DLW assumptions and model equations as well as potential problems with the mouse respiratory chamber data (19).

Similar to our study, Ebbeling et al. (6) reported significant differences in EE_{DLW} during the last two weeks in each of three consecutive month-long periods during which subjects consumed a low-carbohydrate diet (10% of energy) compared to isocaloric diets with moderate (40% of energy) or high (60% of energy) carbohydrate in random order. The lower-carbohydrate diets appeared to increase EE_{DLW} by 250-325 kcal/d compared to the high-carbohydrate diet. However, despite constant energy intake there were no significant weight differences among the three month-long diet periods. Objectively measured physical activity was not different among the diets, and the resting energy expenditure differences were much smaller (~29-67 kcal/d) than the EE_{DLW} differences. Compared to the high- and moderate-carbohydrate diets, the lowest carbohydrate diet had 50% more dietary protein – which is known to be more thermogenic than dietary carbohydrate and fat (20) – and likely contributed to a portion of the observed increase in EE_{DLW}. But it is unlikely that the thermic effect of protein alone could explain the discrepancy

between the differences in resting energy expenditure and EE_{DLW}. Thus, the data by Ebbeling et al. could be interpreted as supporting the possibility that the DLW method is systematically biased by dietary carbohydrate content such that isocaloric diets that are low in carbohydrate content result in higher estimates of energy expenditure.

In contrast, Bandini et al. (21) found that EE_{DLW} was lower during the low-carbohydrate diet (~7% of energy) as compared to a high-carbohydrate diet (~83% of energy) in an outpatient study, but this reduction was attributed to decreased physical activity because no significant differences in REE were found and the subjects reported nausea and lethargy on the low-carbohydrate diet. Stubbs et al. (22) found no significant difference between EE_{DLW} using a narrower range of diets with 29-67% of energy as carbohydrate. But the diets were fed *ad libitum* and energy intake on the low-carbohydrate diet was greater than the moderate-carbohydrate diet which was greater than the high-carbohydrate diet. Thus, the variation in total carbohydrate content of the test diets was attenuated such that daily carbohydrate intake varied by only ~22% of the mean energy intake between diets which may not have been a sufficient range to observe a systematic bias of the DLW method.

It is important to emphasize that our study was not intended to be a DLW validation study and there were several limitations. The subjects lost weight and body fat throughout the study, consistent with a state of inadvertent overall negative energy balance amounting to ~200-400 kcal/d (12). The DLW method has been validated during 30% caloric restriction with a 55% carbohydrate diet (23) and agrees with our result that EE_{DLW} was not significantly different from EE_{bal} during the BD diet phase. Nevertheless, the calculated EE_{bal} values are somewhat uncertain

because DXA has a limited ability to precisely and accurately detect small changes in body energy stores (24), and the calculated metabolizable energy intake was not adjusted using direct measures of fecal energy content. While accelerometer measurements did not detect significant differences in physical activity between the diets, these devices do not capture all forms of physical activity and we cannot rule out the possibility that the KD resulted in undetected increases in activity-related energy expenditure. Finally, the order of the diets was not randomized, and it is possible that the elevated EE_{DLW} occurred simply because the KD followed the BD. Indeed, other investigators have noted that repeated DLW measurements resulted in a mean EE_{DLW} increase of ~100 kcal/d for the second measurement, perhaps for methodological reasons related to the isotopes in the first DLW dose interfering with subsequent measures (Jim DeLany, personal communication).

In summary, our data raise the possibility that the DLW method results in a systematic bias when calculating energy expenditure differences between diets varying widely in the proportion of carbohydrate. We do not suggest that our study is definitive in this regard. A validation study in humans is needed, with several days of simultaneous respiratory chamber and DLW measurements in subjects consuming high- and low-carbohydrate diets. Until such a validation study is conducted, we urge caution when interpreting DLW data comparing energy expenditure differences between diets varying widely in carbohydrate content unless such data can be corroborated by quantitatively commensurate observations of energy intake and body composition.

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Figure Legends

Figure 1. A) Neither energy intake (EI), energy expenditure by respiratory chamber ($EE_{chamber}$), nor energy expenditure by balance (EE_{bal}) were significantly different during the baseline diet (BD) versus the ketogenic diet (KD). However, energy expenditure by doubly labeled water (EE_{DLW}) was significantly greater during the KD. B) Differences in EI, $EE_{chamber}$, EE_{bal}, and EE_{DLW} between KD and BD phases. NS = not significant, $p > 0.05$.

Figure 2. Correlations between individual measurements of A) $EE_{chamber}$ and EE_{bal}, B) $EE_{chamber}$ and EE_{DLW}, and C) EE_{DLW} and EE_{bal} were high during the BD phase. During the KD phase, D) $EE_{chamber}$ and EE_{bal} remained highly correlated, whereas the correlation between E) $EE_{chamber}$ and EE_{DLW} was diminished and F) EE_{DLW} and EE_{bal} were no longer significantly correlated.

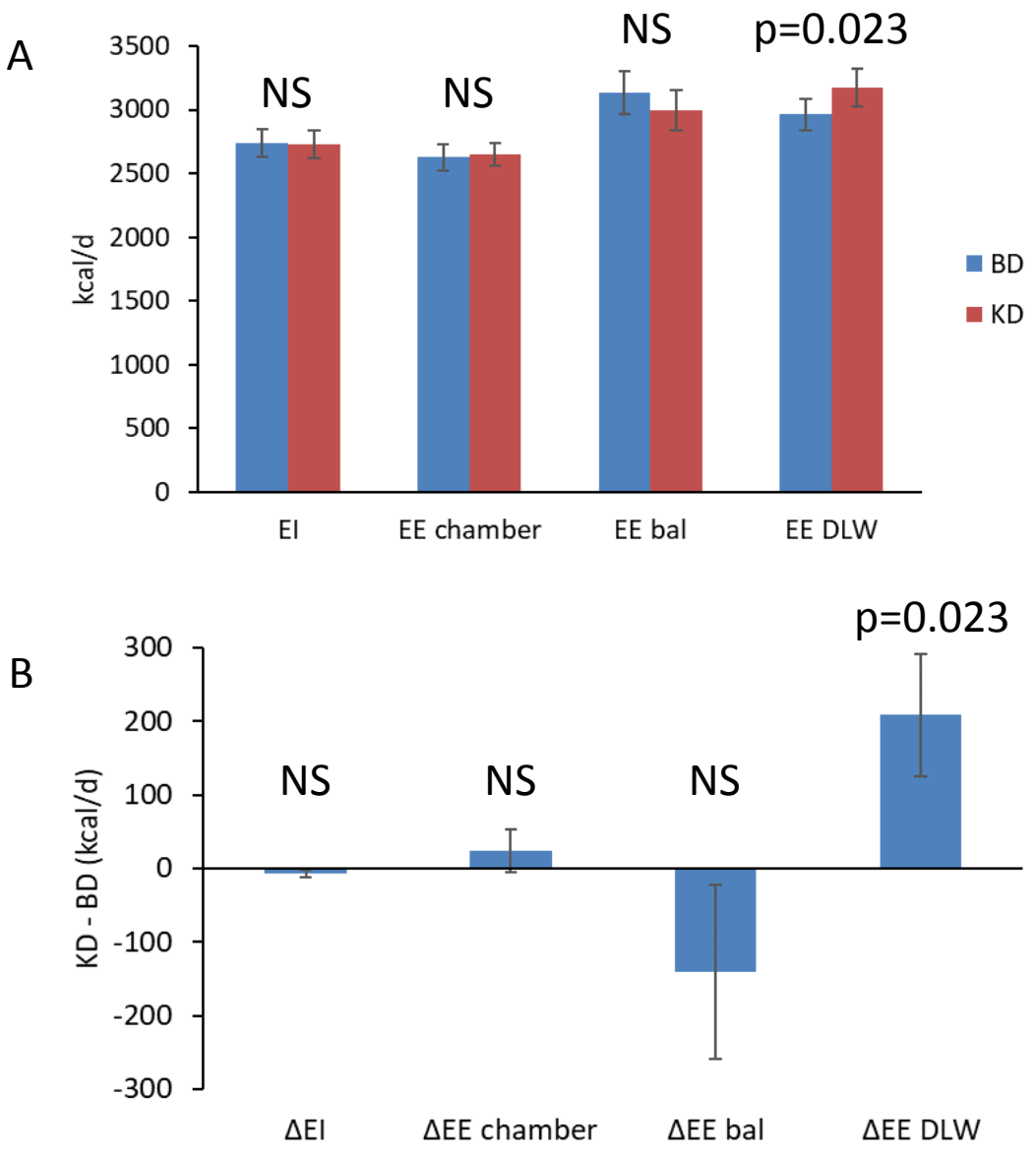
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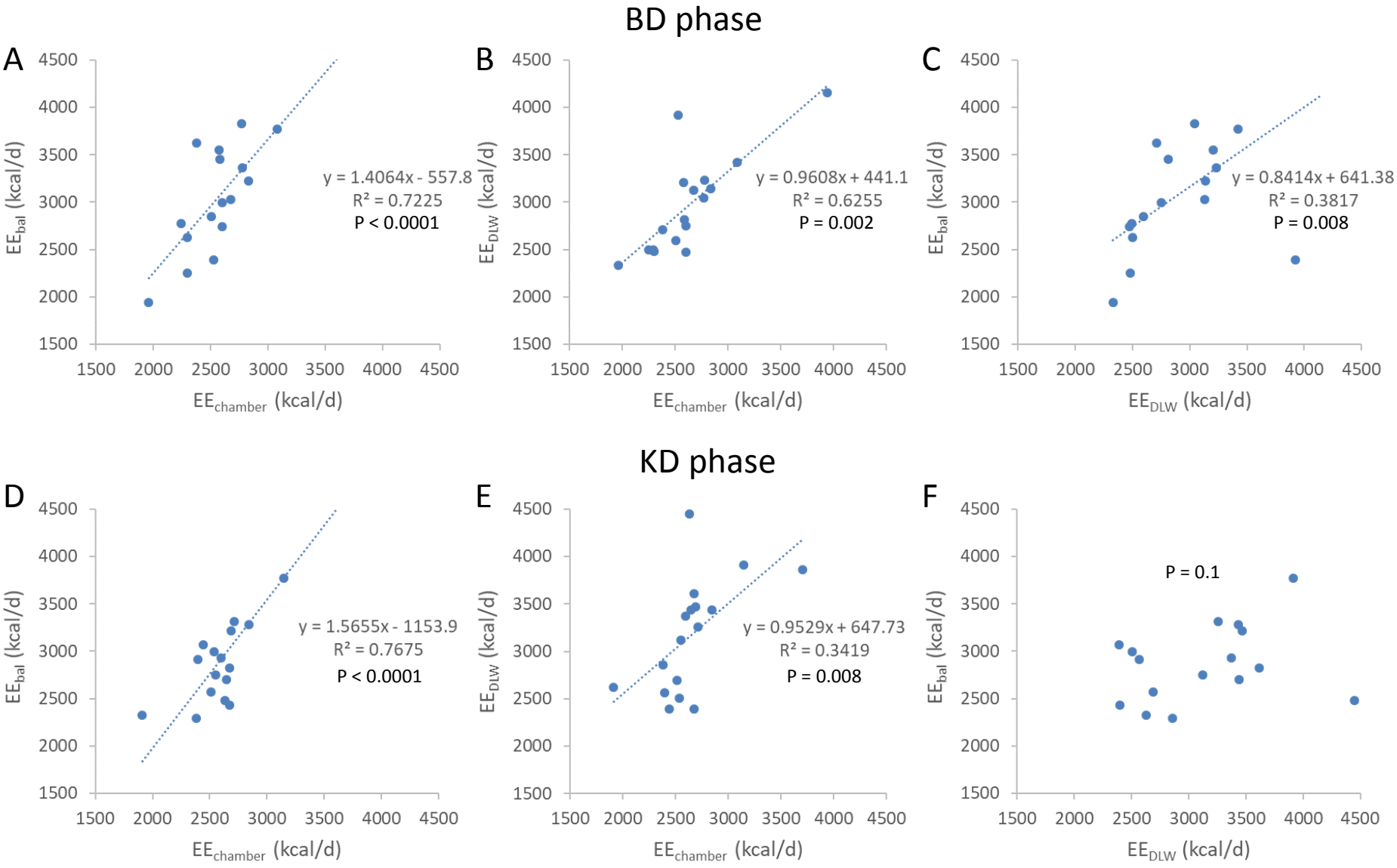
Figure 1


BD phase
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y = 1.4064x - 557.8
R² = 0.7225
P < 0.0001
y = 0.9608x + 441.1
R² = 0.6255
P = 0.002
y = 0.8414x + 641.38
R² = 0.3817
P = 0.008
EE_bal (kcal/d)
EE_DLW (kcal/d)
EE_chamber (kcal/d)
KD phase
D
E
F
y = 1.5655x - 1153.9
R² = 0.7675
P < 0.0001
y = 0.9529x + 647.73
R² = 0.3419
P = 0.008
P = 0.1

Figure 2

Supplementary Materials

We used the two-pool doubly labeled water (DLW) equations of Speakman [1] to calculate the rate of CO_2 production (rCO_2) due to energy expenditure (EE) as follows:

$$r_{CO_2\,EE}\left(\text{mol/d}\right) = (N / 2.078)\left[1.007 \times k_o - 1.007 \times 1.034 \times k_d\right] - 0.0246 \times r_{GF}$$

where N is the body water pool size (in moles), k_O and k_D are the rate constants (in d^{-1}) describing the exponential disappearances of the labeled ^{18}O and deuterated water isotopes in repeated spot urine samples collected over several days, and r_{GF} accounts for the fractionation of the isotopes.

It is known that deuterium is incorporated into body constituents other than water via reductive biosynthesis, and the fluxes through some of these biosynthetic pathways likely depends on diet composition [2-4]. For example, the incorporation of deuterium into newly synthesized fatty acids via de novo lipogenesis (DNL) results in an apparent sequestration of 0.0296 moles of deuterated water per gram of DNL [2] and the flux through the DNL pathway depends highly on dietary carbohydrate. Therefore, during ongoing DNL, the CO_2 production rate measured by DLW is correspondingly altered:

$$r_{CO_2\,meas}\left(\text{mol/d}\right) = (N / 2.078)\left[1.007 \times k_o - 1.007 \times 1.034 \times k_d\right] - 0.0246 \times r_{GF} - \frac{0.0296\left(\text{mol/g}\right)}{2.078} \times DNL\left(\text{g/d}\right)$$

$$r_{CO_2 meas}\left(\text{mol/d}\right) = r_{CO_2 EE}\left(\text{mol/d}\right) - \frac{0.0296\left(\text{mol/g}\right)}{2.078} \times DNL\left(\text{g/d}\right)$$

Therefore, when DNL is ongoing the rate of CO_2 production (and therefore EE) as measured by the DLW method underestimates the rate of CO_2 production. However, parameterization of the

equations employed in the DLW method used data in subjects with ongoing DNL, since they were consuming moderate to high carbohydrate (HC) diets. The potential bias of the DLW method with HC diets was likely offset in the calibration/validation process of developing the DLW model equations. Thus, if such an offset were significant then transitioning to a lower carbohydrate diet (LC) would be expected to result in a decrease in DNL and an apparent increase in EE_{DLW} solely due to a methodological bias.

How much could DNL differences potentially contribute to the EE_{DLW} differences between LC and HC diets? In our study [5], we calculated the energy equivalent of CO_2 as:

$$Eeq_{CO_2}\left(\text{kcal/L}\right)=\frac{3.85}{RQ}+1.07$$

Assuming that the LC diet had negligible DNL (DNL=0) and there was no actual difference in EE between the diets: $Eeq_{CO_2HC}\times r_{CO_2EE_{HC}}=Eeq_{CO_2LC}\times r_{CO_2EE_{LC}}$, then the measured difference in EE_{DLW} between LC and HC due solely to DNL is:

$$\Delta EE_{meas}\left(\text{kcal/d}\right)=22.261\left(\text{L/mol}\right)\left[Eeq_{CO_2HC}\times r_{CO_2meas_{HC}}-Eeq_{CO_2LC}\times r_{CO_2meas_{LC}}\right]$$
$$=-22.261\left(\text{L/mol}\right)\times Eeq_{CO_2HC}\left(\text{kcal/L}\right)\times\frac{0.0296\left(\text{mol/g}\right)}{2.078}\times DNL\left(\text{g/d}\right)$$

Therefore, the increment in kcal/d for every g/d of DNL is:

$$\frac{\Delta EE_{meas}\left(\text{kcal/d}\right)}{DNL\left(\text{g/d}\right)}=-22.261\ \text{(L/mol)}\times Eeq_{CO_2HC}\text{(kcal/L)}\times\frac{0.0296\ \text{(mol/g)}}{2.078}$$

Assuming that RQ is between 0.85 and 0.90 on the HC diet, then the increment in EE kcal/d per DNL g/d ranges between -1.78 to -1.70.

About 20% of subcutaneous adipose TG synthesis on a HC diet is derived from DNL and adipose TG turnover is in the range of 0.7 to 1.5 g/d per kg BW [6]. Hepatic VLDL TG production rate is about 0.7 g/d per kg BW [7] and about 10-20% of the VLDL TG produced daily may be derived from DNL with higher carbohydrate diets [8]. So, about 17-35 g/d is probably a reasonable estimate for whole body DNL for an 80 kg subject during a HC diet, with a likely large degree of individual variability. Therefore, DNL differences between HC and LC diets likely introduced an error in the EE by DLW to result in an apparent ΔEE_{meas} of about 30-60 kcal/d between LC and HC diets.

We cannot rule out the possibility of larger systematic errors without direct measurements of DNL. Furthermore, other biosynthetic pathways may also contribute to diet-dependent systematic errors in the DLW method in the same direction, although likely not to the same extent as DNL. For example, both glycogen and protein synthesis result in incorporation of deuterium [9] and the fluxes through these pathways would be expected to decrease with a LC diet. Overall, these methodological issues introduce potentially important systematic biases when considering the effect of diet differences on EE as measured by DLW.

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Supplementary Table 1

N=17	BD Mean ± SE	KD Mean ± SE	KD-BD Mean ± SE	P-value
EI (kcal/d)	2738±107	2730±110	-8±5	0.16
$EE_{chamber}$ (kcal/d)	2626±104	2650±89	24±30	0.43
EE_{bal} (kcal/d)	3136±171	2995±160	-141±118	0.25
EE_{DLW} (kcal/d)	2964±126	3173±146	209±83	0.023

Supplementary Table 2

N=16 (excluding subject A)	BD Mean ± SE	KD Mean ± SE	KD-BD Mean ± SE	P-value
EI (kcal/d)	2740±114	2732±117	-8±5	0.17
$EE_{chamber}$ (kcal/d)	2632±110	2651±95	19±31	0.55
EE_{bal} (kcal/d)	3182±176	3027±167	-155±125	0.23
EE_{DLW} (kcal/d)	2905±118	3093±130	189±86	0.044

Supplementary Table 3

N=16 (excluding subject B)	BD Mean ± SE	KD Mean ± SE	KD-BD Mean ± SE	P-value
EI (kcal/d)	2736±114	2726±117	-10±5	0.06
$EE_{chamber}$ (kcal/d)	2628±110	2649±95	21±32	0.52
EE_{bal} (kcal/d)	3160±181	3006±170	-155±125	0.24
EE_{DLW} (kcal/d)	2995±130	3146±152	151±63	0.031

Supplementary Table 4

N=15 (excluding subjects A and B)	BD Mean ± SE	KD Mean ± SE	KD-BD Mean ± SE	P-value
EI (kcal/d)	2738±122	2728±125	-10±5	0.064
$EE_{chamber}$ (kcal/d)	2635±118	2650±102	15±33	0.65
EE_{bal} (kcal/d)	3212±185	3041±177	-171±133	0.22
EE_{DLW} (kcal/d)	2933±122	3059±134	126±62	0.062