1   **Methodologic Issues in Doubly Labeled Water Measurements of Energy Expenditure**

2   **During Very Low-Carbohydrate Diets**

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29   **Abstract**

30   **Background:** Very low-carbohydrate diets have been reported to substantially increase human

31   energy expenditure as measured by doubly labeled water (DLW) but not by respiratory

32   chambers. Do the DLW data reflect true physiological differences that are undetected by

33   respiratory chambers? Alternatively, are the apparent DLW energy expenditure a consequence of

34   failure to fully account for respiratory quotient (RQ) differences between diets?

35   **Objective:** To examine energy expenditure differences between diets varying drastically in

36   carbohydrate and to quantitatively compare DLW data with respiratory chamber and body

37   composition measurements within an energy balance framework.

38   **Design:** DLW measurements were obtained during the final two weeks of month-long baseline

39   (BD; 50% carbohydrate, 35% fat, 15% protein) and isocaloric ketogenic diets (KD; 5%

40   carbohydrate, 80% fat, 15% protein) in 17 men with BMI 25-35 kg/m^2. Subjects resided 2d/week

41   in respiratory chambers to measure energy expenditure ($EE_{chamber}$). DLW expenditure was

42   calculated using chamber-determined respiratory quotients (RQ) either unadjusted (EE_{DLW}) or

43   adjusted ($EE_{DLW\Delta RQ}$) for net energy imbalance using diet-specific coefficients. Accelerometers

44   measured physical activity. Body composition changes were measured by dual-energy X-ray

45   absorptiometry which were combined with energy intake measurements to calculate energy

46   expenditure by balance (EE_{bal}).

47   **Results:** After transitioning from BD to KD, neither $EE_{chamber}$ nor EE_{bal} were significantly

48   changed ($\Delta EE_{chamber}=24\pm30$ kcal/d; p=0.43 and $\Delta EE_{bal}=-141\pm118$ kcal/d; p=0.25). Similarly,

49   physical activity (-5.1±4.8%; p=0.3) and exercise efficiency (-1.6±2.4%; p=0.52) were not

50   significantly changed. However, EE_{DLW} was 209±83 kcal/d higher during the KD (p=0.023) but

51   was not significantly increased when adjusted for energy balance ($EE_{DLW\Delta RQ}$ =139±89 kcal/d;

52   p=0.14). After removing 2 outliers whose EE_{DLW} were incompatible with other data, EE_{DLW} and

53    $EE_{DLW\Delta RQ}$ were marginally increased during the KD by 126±62 kcal/d (p=0.063) and 46±65

54   kcal/d (p=0.49), respectively.

55   **Conclusions:** DLW calculations failing to account for diet-specific energy imbalance effects on

56   RQ erroneously suggest that very low carbohydrate diets substantially increase energy

57   expenditure.

58

59 **Introduction**

60 There is a great deal of interest in whether very low-carbohydrate diets offer a "metabolic

61 advantage" for weight loss by increasing total energy expenditure (1, 2) – a phenomenon

62 predicted by the carbohydrate-insulin model of obesity (3, 4). In support of this concept, a

63 highly-cited controlled feeding study in humans using the doubly labeled water (DLW) method

64 found a substantial increase in average daily energy expenditure during a very low-carbohydrate

65 diet compared with an isocaloric high-carbohydrate diet (5). However, such results are

66 inconsistent with several controlled feeding studies employing respiratory chambers that failed

67 to detect important energy expenditure differences between isocaloric diets varying in

68 carbohydrate content (6-8).

69

70 Our recent controlled feeding inpatient study was the first to compare daily energy expenditure

71 between isocaloric diets widely varying in carbohydrate content using both respiratory chambers

72 and DLW (9). Our primary result was a small but statistically significant increase daily energy

73 expenditure as measured by respiratory chamber ($EE_{chamber}$) of 57 ± 13 kcal/d (P = 0.0004) after

74 17 men ($25<BMI<35$ kg/m^2) transitioned from a one-month inpatient run-in period consuming a

75 moderate-carbohydrate baseline diet (BD) (50% carbohydrate, 35% fat, 15% protein) to a

76 subsequent month-long inpatient period consuming an isocaloric ketogenic diet (KD) (5%

77 carbohydrate, 80% fat, 15% protein). An exploratory aim of our study was to measure changes in

78 average daily energy expenditure by the DLW method (EE_{DLW}) and we reported a more

79 substantial increase of 151 ± 63 kcal/d (P = 0.03 uncorrected for multiple comparisons)

80 following the KD (9).

81

82   Some investigators who promote the carbohydrate-insulin model of obesity have discounted

83   these $EE_{chamber}$ results and have reinterpreted our exploratory EE_{DLW} data as underestimating the

84   true physiological effect of very low carbohydrate diets which have been suggested to increase

85   energy expenditure by 200-300 kcal/d (10, 11). However, there are methodological reasons why

86   the energy expenditure calculated by the DLW method is impacted by dietary carbohydrate

87   content that do not reflect true physiological differences. Specifically, the DLW method requires

88   an estimate of the overall metabolic fuel utilization of the body as quantified by the average daily

89   respiratory quotient (RQ). While it is widely known that RQ depends on the composition of the

90   diet as well as the overall state of energy balance, it is less well appreciated that the energy

91   imbalance adjustment of RQ also depends on dietary carbohydrate (12) and such adjustments are

92   rarely employed in DLW calculations.

93

94   Here, we reanalyzed our DLW data taking account of the diet-specific energy imbalance

95   adjustments of RQ used in the DLW method and highlight some key challenges to interpreting

96   DLW data when comparing energy expenditure differences between diets varying in

97   carbohydrate.

98

99   **Methods**

100   Details of the study and its approval by the Institutional Review Boards were reported previously

101   (9). Briefly, 17 men with BMI between 25-35 kg/m^2 provided informed consent and were

102   admitted as inpatients to metabolic wards where they consumed a standard baseline diet (BD)

103   composed of 50% energy from carbohydrate, 35% fat, and 15% protein for 4 weeks immediately

104   followed by 4 weeks of an isocaloric very low-carbohydrate, ketogenic diet (KD) composed of

105   5% carbohydrate, 80% fat, 15% protein. Body weight and height were measured to the nearest

106   0.1 kg and 0.1 cm, respectively, with subjects wearing a hospital gown and undergarments and

107   following an overnight fast. Body fat was measured using dual-energy X-ray absorptiometry

108   (DXA) scanners (Lunar iDXA, GE Healthcare, Madison, WI, USA).

109

110   Subjects spent two consecutive days each week residing in respiratory chambers to measure

111   energy expenditure ($EE_{chamber}$). As described previously (9), during the BD period, the daily

112   energy expenditure was calculated as follows:

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

114   where VO_2 and VCO_2 were the volumes of oxygen consumed and carbon dioxide produced,

115   respectively, and N was the 24hr urinary nitrogen excretion measured by chemiluminescence

116   (Antek MultiTek Analyzer, PAC, Houston, TX). During the KD period, the equations were

117   adjusted to account for 24-hour urinary ketone excretion, K_{excr}:

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

119

120   Energy efficiency of physical activity was measured in the respiratory chamber with subjects

121   exercising at a constant, self-selected, level of moderate-intensity cycle ergometry.

122

123   Energy expenditure was calculated by energy balance (EE_{bal}) using the daily metabolizable

124   energy intake (EI) and the measured rates of change of the body energy storage pools determined

125   from measurements of fat mass (FM) and fat-free mass (FFM) by DXA at the beginning and end

126   of each two-week BD and KD period coincident with the DLW measurements as follows:

127     $EE_{bal} = EI - \rho_{FFM}\dfrac{dFFM}{dt} - \rho_{FM}\dfrac{dFM}{dt}$

128   where ρ_{FM} = 9300 kcal/kg is the energy density of body fat mass, ρ_{FFM} = 1100 kcal/kg is the

129   energy density of fat-free mass.

130

131   Energy expenditure was measured by DLW during the final two weeks of the BD and KD

132   periods to allow sufficient time for fluid shifts as subjects adjusted to each diet. Subjects drank

133   from a stock solution of ^2H_2O and $H_2^{18}O$ water in which 1 g of ^2H_2O (99.99% enrichment) was

134   mixed with 19 g of $H_2^{18}O$ (10% enrichment). An aliquot of the stock solution was saved for

135   dilution to be analyzed along with each set of urine samples. The water was weighed to the

136   nearest 0.1 g into the dosing container. The prescribed dose was 1.0 g per kg body weight and

137   the actual dose amounts were entered in the dose log. Spot urine samples were collected daily.

138   Isotopic enrichments of urine samples were measured by isotope ratio mass spectrometry. The

139   average CO_2 production rate (rCO_2) was corrected for previously administered isotope doses

140   (13), can be estimated from the rate constants describing the exponential disappearances of the

141   labeled ^{18}O and deuterated water isotopes (k_O and k_D) in repeated spot urine samples collected

142   over several days. We used the parameters of Racette et al. (14) with the weighted dilution space

143   calculation, R_{dil}, proposed by Speakman (15):

$$rCO_2 = \left(N/2.078\right)\left(1.007k_O - 1.007R_{dil}k_D\right) - 0.0246r_{GF}$$

144    $$r_{GF} = 1.05\left(1.007k_O - 1.007R_{dil}k_D\right)$$

$$R_{dil} = \left[\left(N_D/N_O\right)_{ave} \times n + 1.034 \times 255\right]/\left(n + 255\right)$$

145   where r_{GF} accounts for the fractionation of the isotopes and $(N_D / N_O)_{ave}$ is the mean of the $N_D /$

146   N_O values from the $n=17$ subjects.

147

148   In our previous report (9), we used the 24hr respiratory quotient, RQ, measured during the

149   respiratory chamber stays to calculate energy expenditure (EE_{DLW}) during the baseline period

150   was calculated as:

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

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

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

154

155   However, because the energy expended outside the chamber was significantly greater than inside

156   the chamber (9), the chamber RQ values did not accurately represent the overall RQ values

157   during the DLW period. In other words, the relative magnitude of energy imbalance (EB) during

158   the DLW period was different than the energy imbalance (EI-$EE_{chamber}$) measured during the

159   chamber stays. Therefore, we used the overall energy imbalance during the DLW period, defined

160   by the rate of change of body energy stores, to adjust the chamber RQ measurements by an

161   amount ΔRQ as described in the Online Supplemental Materials:

162    $\Delta RQ = \lambda \times EB$

163   where $\lambda = 5.28 \times 10^{-5}$ d/kcal for the BD and $\lambda = 9.17 \times 10^{-6}$ d/kcal for the KD.

164

165   Importantly, the value of λ is affected by dietary carbohydrate content, and, consequently, the

166   energy equivalence of CO_2 produced in the DLW calculations differs between diets varying in

167   carbohydrate for the same degree of energy imbalance.

168

169   Thus, the ΔRQ adjustment for each individual's diet-specific state of energy imbalance results in

170   the following calculation of DLW energy expenditure ($EE_{DLW\Delta RQ}$) during the baseline period:

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

172   During the KD period, $EE_{DLW\Delta RQ}$ was calculated as:

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

174

175   As opposed to our previous study (9) that reported results during the entire six-week period when

176   EI was held constant (i.e. the last two weeks of the BD phase and the entirety of the KD phase),

177   we now report results based upon data obtained only during the two-week DLW phase of both

178   BD and KD periods. In addition to allowing direct comparison with the coincident DLW

179   measures, focusing on the last two weeks of each diet allowed for two weeks to adapt to the BD

180   from the usual diet as well as to the KD from BD.

181

182   Statistical analysis was performed using a paired, two-sided t-test with significance declared at

183   the $p < 0.05$ threshold. The data are reported as mean±SE.

184

185   **Results**

186   During the final two weeks of the BD, EI was 2738±107 kcal/d which was significantly higher

187   than $EE_{chamber}$ = 2626±104 kcal/d ($p < 0.0001$). EE_{DLW} was 2964±126 kcal/d and significantly

188   higher than EI ($p = 0.011$). After adjusting for the energy imbalance, $EE_{DLW\Delta RQ}$ = 3045±135

189   kcal/d was significantly greater than EE_{DLW} ($p = 0.0003$). Energy expenditure calculated using EI

190   and the changes in body energy stores was EE_{bal} = 3136±171 kcal/d and was not significantly

191   different from EE_{DLW} (p=0.23) or $EE_{DLW\Delta RQ}$ (p=0.47). Compared to $EE_{chamber}$, EE_{DLW} was

192   338±77 kcal/d higher (p=0.0005), $EE_{DLW\Delta RQ}$ was 419±76 kcal/d higher (p<0.0001), and EEbal

193   was 509±100 kcal/d higher (p<0.0001) indicating that subjects expended significantly more

194   energy outside the chamber during the BD phase.

195

196   During the final two weeks of the KD phase, EI was 2730±110 kcal/d which, by design, was not

197   significantly different from the BD phase (p=0.16). Whereas we previously reported a transient

198   increase in $EE_{chamber}$ during the first two weeks after introducing the KD (9), neither $EE_{chamber}$

199   (2650±89 kcal/d; p=0.43) nor EE_{bal} (2995±160 kcal/d; p=0.25) were significantly different

200   during the last two weeks of the KD compared to the last two weeks of the BD coincident with

201   the DLW measurement periods (Figure 1A). Likewise, physical activity measured using an

202   accelerometer mounted on the hip was not significantly different (KD relative to BD, -5.1±4.8%;

203   p=0.3); and energy efficiency of physical activity measured in the respiratory chamber with

204   subjects exercising at a constant level of moderate-intensity cycle ergometry was not

205   significantly different (-1.6±2.4%; p=0.52) between the BD and KD phases.

206

207   Despite no significant differences in EI, $EE_{chamber}$, EE_{bal}, physical activity, or exercise efficiency

208   between the KD and BD phases (Figure 1B), EE_{DLW} was 209±83 kcal/d higher during the KD

209   phase (p=0.023). The transition from the BD to KD coincided with increases in EE_{DLW} that were

210   in the opposite direction to EE_{bal}, indicating that the DLW calculations during the KD were

211   incommensurate with the changes in body weight and fat mass. After adjusting for the degree of

212   energy imbalance, $EE_{DLW\Delta RQ}$ was 139±89 kcal/d higher during the KD, but this difference was

213   no longer significant (p=0.14) and was still in a direction opposite to the changes in body energy

214   stores.

215

216   There were two clear DLW outliers. The first outlier, "subject A", had an EE_{DLW} that was 1220

217   kcal/d greater than EI during the BD, and was 1751 kcal/d greater than EI during the KD despite

218   slight body weight and fat mass gains during these periods. In contrast, the $EE_{chamber}$

219   measurements for this subject were 173 kcal/d less than EI during the BD, and 65 kcal/d less

220   than EI during the KD. The second outlier, "subject B", had an EE_{DLW} during the BD that was

221   only 123 kcal/d higher than $EE_{chamber}$, but during the KD his EE_{DLW} increased by 1136 kcal/d

222   which was ~3 standard deviations greater than the mean increase in EE_{DLW}, suggesting severe

223   negative energy balance despite the subject gaining weight during this period and an $EE_{chamber}$

224   increasing by only 72 kcal/d. Tables S1-S4 in the Online Supplemental Materials provide

225   summary data on the energy expenditure comparisons between BD and KD phases with and

226   without the exclusion of these subjects. After excluding these subjects, the increase in EE_{DLW}

227   during the KD was 126±62 kcal/d (p=0.063) and $EE_{DLW\Delta RQ}$ increased by only 46±65 kcal/d

228   (p=0.49), neither of which were significant (Table S4)

229

230   **Discussion**

231   As previously reported (9), our inpatient isocaloric feeding study showed that four days of

232   respiratory chamber measurements that were coincident with each two-week DLW period did

233   not detect significant changes in energy expenditure that were attributable to diet composition.

234   However, the increase in EE_{DLW} after transitioning to the KD was substantially greater than

235   could be accounted for by changes in body energy stores. In the present analysis, after employing

236   diet-specific RQ adjustments in the DLW calculations to account for energy imbalance (12), we

237   found that the calculated increase in DLW energy expenditure during the KD was significantly

238   attenuated and was no longer statistically significant. After removal of two clear DLW outliers,

239   the RQ-adjusted increase in DLW energy expenditure was further reduced to a marginal ~50

240   kcal/d higher during the KD.

241

242   We previously hypothesized that the discrepancy between the respiratory chamber and DLW

243   measurements was due to increased physical activity outside the respiratory chamber during the

244   KD (9). However, this potential explanation does not agree with the lack of increase in

245   objectively measured physical activity. Also, the energy expended to perform the same low-

246   intensity exercise in the respiratory chamber was not significantly changed by the KD, making it

247   unlikely that the energy efficiency of skeletal muscle contraction had been altered after

248   transitioning to the KD. Thus, the EE_{DLW} measurements were discordant with several

249   independent measures indicating that the KD did not result in significant energy expenditure

250   changes. The appropriately adjusted RQ values resulted in DLW data more commensurate with

251   the other data, especially after removing the pair of outliers.

252

253   Another methodological concern with the DLW method is the theoretical possibility that CO_2

254   production rates can be influenced by the fluxes through biosynthetic pathways that likely vary

255   substantially depending on the carbohydrate content of the diet, especially the de novo

256   lipogenesis pathway (16-18). However, the magnitude of this potential bias in humans is thought

257   to be relatively small, amounting to an energy expenditure difference of only about 30-60 kcal/d

258   (see Online Supplemental Materials). Interestingly, even this small systematic bias would have

259   been sufficient to nullify the statistical significance of the observed increased EE_{DLW} in our study

260   and the effect size was similar to the nonsignificant increase in the $EE_{DLW\Delta RQ}$ difference after

261   removal of the outliers. Individual variability of de novo lipogenesis changes after transitioning

262   to the KD might explain the diminished correlations during the KD between the DLW

263   measurements with $EE_{chamber}$ and EE_{bal}. To directly address this question, we need a validation

264   study in humans with several days of simultaneous respiratory chamber and DLW measurements

265   high- and low-carbohydrate diets, ideally with simultaneous measurements of de novo

266   lipogenesis.

267

268   Ebbeling et al. (5) reported significant ~200-300 kcal/d differences in EE_{DLW} during the last two

269   weeks in each of three consecutive month-long periods during which outpatient subjects

270   consumed a very low-carbohydrate diet (10% of energy) compared to isocaloric diets with

271   moderate (40% of energy) or high (60% of energy) carbohydrate in random order. However,

272   resting energy expenditure differences were much smaller (~29-67 kcal/d) and objectively

273   measured physical activity was not significantly different among the diets. Despite claims of

274   weight stability, reported energy intake was several hundred calories below the measured EE_{DLW}

275   but RQ was estimated without adjustment for the subjects' state of energy balance. Therefore,

276   similar to the current study, the diet specific effects of energy imbalance on RQ likely

277   contributed to the observed differences in EE_{DLW}. Thus, the data of Ebbeling et al. could be

278   interpreted as supporting the possibility that the EE_{DLW} calculations using RQ values unadjusted

279   for energy imbalance in a diet-specific way were systematically biased such that the isocaloric

280   very low-carbohydrate diet resulted in systematically increased EE_{DLW}.

281

282   In contrast, Bandini et al. (19) found in an outpatient study that EE_{DLW} was lower during the very

283   low-carbohydrate diet (~7% of energy) as compared to a high-carbohydrate diet (~83% of

284   energy), but this reduction was attributed to decreased physical activity because the subjects

285   reported nausea and lethargy on the low-carbohydrate diet. No significant differences in REE

286   were found. Stubbs et al. (20) found no significant difference between EE_{DLW} using a narrower

287   range of diets with 29-67% of energy as carbohydrate. But the diets were fed *ad libitum* and

288   energy intake on the low-carbohydrate diet was greater than the moderate-carbohydrate diet

289   which was greater than the high-carbohydrate diet. Thus, the variation in total carbohydrate

290   content of the test diets was attenuated such that daily carbohydrate intake varied by only ~22%

291   of the mean energy intake between diets which may not have been a sufficient range to observe a

292   systematic bias of the DLW method. Furthermore, the positive energy balance with the low-

293   carbohydrate diet increased RQ whereas the negative energy balance with the high-carbohydrate

294   diet decreased RQ. Thus, the RQ differences during consumption of these diets were attenuated

295   by the differences in energy balance.

296

297   It is important to emphasize that our study was not intended to be a DLW validation study and

298   there were several limitations. The DLW measurements were not pre-specified as either primary

299   or secondary endpoints of the study. Whereas respiratory chamber measurements have high-

300   precision, with an intrasubject coefficient of variation of $EE_{chamber}$ ~2-3% (21), the DLW method

301   is less precise, with an intrasubject coefficient of variation of energy expenditure of ~ 8-15%

302   (22). Therefore, the relatively large inherent variability of the DLW method may have led to an

303   apparent increase in EE_{DLW} during the KD simply by chance (type-1 error). However, we cannot

304   definitively exclude the possibility of a real increase in energy expenditure, especially at an

305   effect size of ~50-140 kcal/d after excluding two likely DLW outliers or using diet-specific

306   adjustments of the DLW calculations to account for the energy imbalance.

307

308   The DLW method has been validated during 30% caloric restriction with a 55% carbohydrate

309   diet (23) and agrees with our result that EE_{DLW} and $EE_{DLW\Delta RQ}$ were not significantly different

310   from EE_{bal} during the BD diet phase. Nevertheless, the calculated EE_{bal} values are somewhat

311   uncertain because DXA has a limited ability to precisely and accurately detect small changes in

312   body energy stores (24). We cannot rule out the possibility that the KD resulted in undetected

313   increases in activity-related energy expenditure that were undetected by accelerometers. Finally,

314   the order of the diets was not randomized, and it is possible that the elevated EE_{DLW} occurred

315   simply because the KD followed the BD. Indeed, others have reported a greater metabolic rate

316   during a low-carbohydrate diet when it followed a high-carbohydrate diet as compared with the

317   reverse order (25).

318

319   In summary, our data illustrate the challenges of using the DLW method to estimate energy

320   expenditure differences between diets varying widely in the proportion of carbohydrate. We urge

321   caution when interpreting such data, especially if the DLW calculations do not appropriately

322   adjust RQ for energy imbalance or if the DLW results are not corroborated by quantitatively

323   commensurate observations of energy intake and body composition change.

324

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330   SRS, and ER designed the study and conducted the research; KDH and JG analyzed the data;

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332   responsibility for the final content. The authors have no conflicts of interest. We thank Jim

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336   for any purpose by anyone.

337

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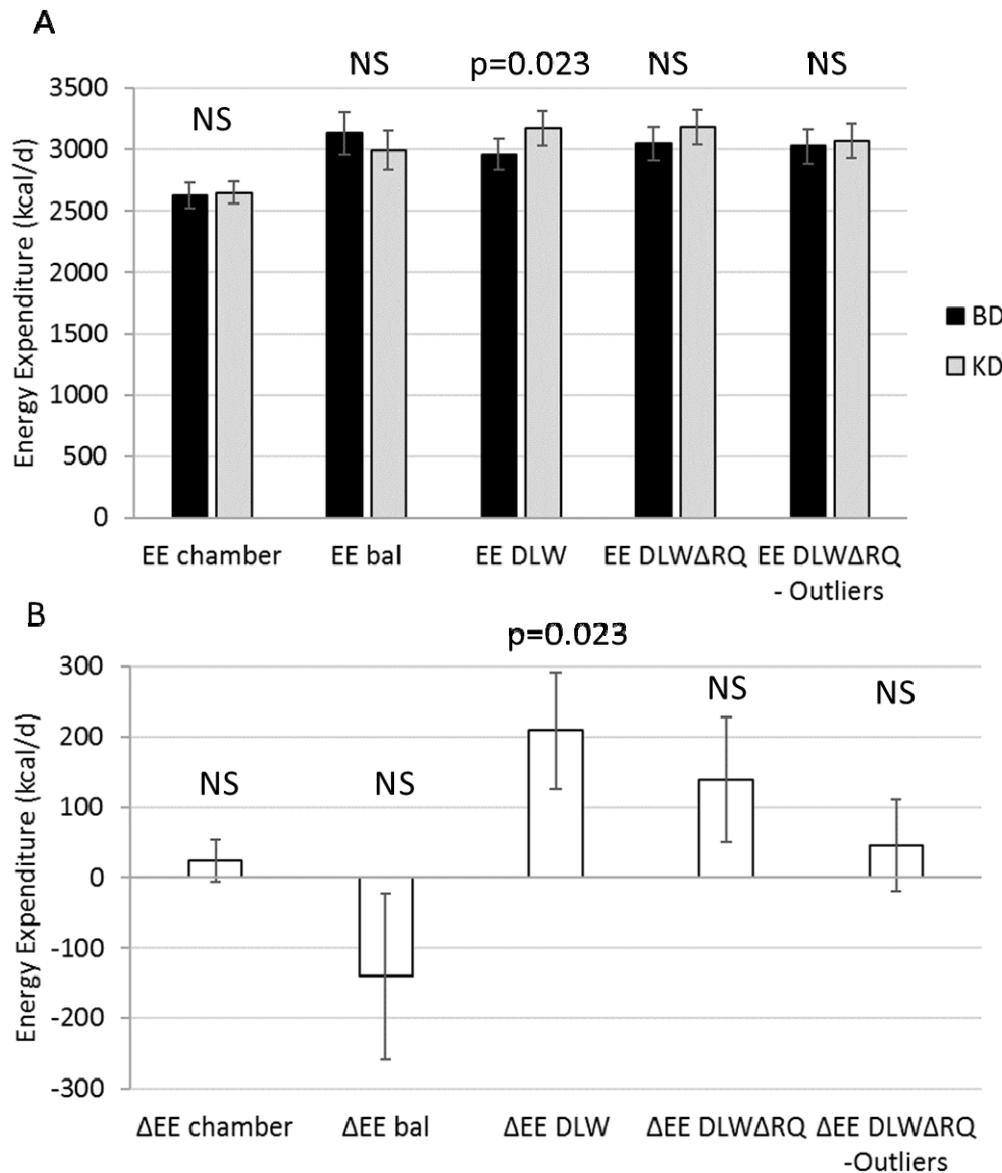407   Spring) 2015;23(11):2216-22. doi: 10.1002/oby.21213.
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Figure 1. A) Neither energy expenditure by respiratory chamber ($EE_{chamber}$), nor energy expenditure by balance (EE_{bal}) were significantly different during the baseline diet (BD) versus the ketogenic diet (KD). However, energy expenditure by doubly labeled water (EE_{DLW}) was significantly greater during the KD, but not after adjustment of the respiratory quotient (RQ) to account for the differential diet effect of energy imbalance ($EE_{DLW\Delta RQ}$) or after removal of two outliers whose DLW data were incomparible with other measurements ($EE_{DLW\Delta RQ}$ - Outliers). B)

417   Differences in $EE_{chamber}$, EE_{bal}, EE_{DLW}, $EE_{DLW\Delta RQ}$, and $EE_{DLW\Delta RQ}$ - Outliers between KD and BD

418   phases. NS = not significant, $p > 0.05$.