Validity of doubly labeled water in obese subjects: questioning the validity of any technique requires an indisputable accuracy of the reference method

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TO THE EDITOR: In a recent article, Guidotti et al. (5) reported that the nutritional and body adiposity status affects the dilution space ratio (Nd/No) between deuterium (2H) and 18O and validity of CO2 production (rCO2) by the doubly labeled water (DLW) method in mice. Their data disagree with those reported by many independent research groups.

First, theirs is the only validation study of DLW ever to find that the two-pool model fits the data better than a single-pool model in small animals (<1 kg), and thus we have to question the general validity of their findings. Speakman (21) listed 22 validation studies of the DLW method for mammals and 18 for birds and strongly concluded that a single-pool model fits the data better in small animals (<1 kg). This conclusion was supported by more recent studies that also found that the one-pool model [see Eq. 7.17 in Speakman (21)] fits the data better than two-pool evaluations. These include 1) Visser and Schekkerman (26) and Visser et al. (25), who examined the DLW method in several birds (black-tailed godwit, northern lapwing, and Japanese quail), with relative growth rates ranging from −16.9 to +23.8% on day 1 (27.7–294.4 g) against the rCO2 measured by indirect calorimetry (rCO2IC); 2) Blanc et al. (1) in rats (290 ± 27 g) during isolation and simulated microgravity against rCO2IC; 3) Van Trigt et al. (24) in Japanese quail (~250 g) at different water fluxes against rCO2IC; 4) Gessaman et al. (3a) in growing poultry chicks against rCO2IC; 5) Speakman and Kröl (22) in field voles (~38 g) at 8°C against metabolizable energy intake; and 6) Shirai et al. (20) in rhinoceros auklet (~530 g) against rCO2IC. Therefore, the Guidotti et al. (5) data sit at odds with all prior validation studies of the method, yet surprisingly, they did not mention this, or even discuss possible reasons for this discrepancy.

We are concerned about the accuracy of the indirect calorimetry data reported by Guidotti et al. (5). They reported that the 3-day respiratory quotients (RQ) were 1.01 ± 0.01 (means ± SE) and 0.92 or 0.89 ± 0.01 for regular laboratory chow (13% fat, 63% starch and 24% protein) and high-fat diet (45% fat, 18% starch, 13% sucrose, 24% protein), respectively. These are surprisingly high compared with the previous observations in mice, other mammals, and humans (e.g., see Refs. 6, 10, and 19). RQ >1.00 cannot be observed unless net neolipogenesis is occurring. When the food quotient (FQ), defined as the ideal diet-specific ratio of V˙CO2 to V˙O2, was calculated for each diet using the equation 0.835 × %protein + (1.0 × %carbohydrate) + (0.71 × %fat), the FQs were 0.92 and 0.83 for the regular chow and high-fat diet, respectively. Therefore, there is a large discrepancy between the RQ and FQ, which is particularly surprising after 6 wk of treatment, and therefore, these discrepancies raise doubts over the validity of the indirect calorimetry performed. There is no detail in the article of the methods by which the indirect calorimetry system was calibrated or validated by, for example, alcohol burns. If the accuracy of their use of the reference method is questionable, this may explain why their results are so discrepant from the rest of the literature.

A third concern is that Guidotti et al. (5) report that the nutritional and body adiposity status affects dilution space ratio (Nd/No), especially because 2H incorporation into C-H bonds has been used in biomedical sciences to measure de novo lipogenesis (23). However, such a process does not increase the dilution space ratio, as this ratio reflects rapid exchange of tracer into nonaqueous spaces. Incorporation of tracer into molecules due to their synthesis during the study changes the elimination rate, causing it to be artificially larger than the water elimination (8). Moreover, Matthews and Gilker (11) showed that exchange onto lipids is likely to be negligible, as the C-H bond is not in equilibrium exchange, and only the terminal −OH bond can engage in exchange equilibrium reactions. Hence, one would anticipate that the isotope dilution space would be only marginally greater in facilitated lipogenesis.

In this regard, we conducted a study in 12 wild-type Japanese rabbits (3.42 ± 0.14 kg) with regular control chow (12% fat, 65% carbohydrate, and 23% protein) and following a 16-wk high-fat diet (34% fat, 49% carbohydrate, and 17% protein). The Nd/No was calculated in a period of regular control chow and in the 15th wk of a high-fat diet. Therefore, there is a large discrepancy between the RQ and FQ, which is particularly surprising after 6 wk of treatment, and therefore, these discrepancies raise doubts over the validity of the indirect calorimetry performed. There is no detail in the article of the methods by which the indirect calorimetry system was calibrated or validated by, for example, alcohol burns. If the accuracy of their use of the reference method is questionable, this may explain why their results are so discrepant from the rest of the literature.

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samples obtained at 3 h after dose in Fig. 2 of their article. We are also very confused by the N_{D}/N_{O} presented by Guidotti et al. (5), because in the text the authors state that the N_{D}/N_{O} ranged from 0.99 to 1.04 for the plateau method, but the group means of N_{D}/N_{O} shown in Fig. 2 of their article vary from 1.01 to 1.06. Thus, the interpretation of the figure and the result related to this figure is very difficult. Most importantly, even if it is correct, the large decrease (approximately −0.021) in N_{D}/N_{O} in the obese mice is very difficult to understand and is specifically not explained by elevated exchange onto lipids or increased lipogenesis. Guidotti et al. (5) mentioned ketone body formations during high-fat diet as an explanation of their results, yet confusingly, if they believed this to be the case they did not correct their indirect calorimetry data for such an effect (3). One possible explanation for the dilution space data of Guidotti et al. (5) is that there is an artifact in the isotope abundance measurement. This was shown to be the explanation of dilution spaces outside the range typically reported in the past (17). Guidotti et al. (5) cite a methods paper where the authors use a large memory adjustment method during their analysis (4). Such adjustments can be subject to artifacts by changes in the performance of the system when sample matrices are those other than water.

Fourth, they compared N_{O} and total body water assessed by carcass analysis in high-fat diet mice. However, drying the carcass at 104°C for 4 h is insufficient to get rid of all the water, and this high temperature may also cause volatilization of lipids. Standard methods suggest that drying at 60°C for 14 days is necessary (7, 16). This probably explains the overestimate by DLW. It is also disconcerting that they showed only the result of carcass analysis in high-fat groups and not the control diet data. In addition, the equivalent time of isotope equilibration is correlated with body size of subjects and has generally been seen in <1 h in mice (7, 21). They showed that the plateau was obtained between 2 and 3 h, which disagrees with the previous literature. Yet again, surprisingly, they did not mention this or discuss possible reasons for this discrepancy.

Finally, they extrapolated the result of their study of mice into human research. Even if their work on mice is correct, which in light of the above evidence seems doubtful, small mammals (<1 kg) have different kinetics of stable isotopes and include different assumptions from large mammals (>10 kg), including humans (21). It is well known that the single-pool model overestimates rCO_{2} in large animals (>10 kg), including humans, and the two-pool model of Schoeller et al. (18), with the revised dilution space constant of Racette et al. (14), fits validation data better than the single-pool model. In this respect, there have been validations before in people with obesity, and they show that the DLW method does not generate a biased estimate of CO_{2} production (2, 15).

In conclusion, the study by Guidotti et al. (5) deviates significantly from the weight of evidence established across all previous validation studies, now exceeding more than 50 articles. We suspect that the result is probably specific to their own laboratory, and the methods used therein, rather than to the DLW method in general.

DISCLOSURES
No financial conflicts of interest are declared by the authors.

AUTHOR CONTRIBUTIONS
Y.Y., S.B., and D.A.S. drafted the manuscript; Y.Y., S.B., Y.N., K.N., T.S., and D.A.S. edited and revised the manuscript; Y.Y., S.B., Y.N., K.N., N.E., T.S., and D.A.S. approved the final version of the manuscript; Y.N. and K.N. performed the experiments.

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