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Reply to Yamada et al.: questions and answers to the validity of the doubly labeled water method in high-fat and sucrose-feeding mice irrespective of obesity proneness

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REPLY: we have reported recently that 1) mice fed a diet rich in saturated fat and sugar (HFS), irrespective of whether they are obesity prone or resistant, have an overestimated rate of CO₂ production (rCO₂) when calculated by the doubly labeled water (DLW) method compared with actual gas exchange using indirect calorimetry (IC) equipment and 2) a two-pool model approach, depending on the timing of sampling the initial and the final, showed the least discrepancies for calculating rCO₂ (8). These findings are challenged in a letter to the editor by Yamada et al. (25). A key issue in our debated study (8), which was not addressed at all in the letter by Yamada et al. (25), is that we did not find inconsistencies in rCO₂ between the two methods in mice fed a low-fat (LF) diet (8). Although this is one indication that our methodology is sound, it is possible that the overestimation of rCO₂ in the HFS condition is applicable only to mice. But what if it were applicable to larger animals, including humans, too? In light of these findings, we concluded that “caution needs to be addressed when using the DLW method in humans and animals feeding a HF diet, because MR may be overestimated” (8). Below, we give a point-by-point reaction to the issues raised by Yamada et al. (25).

The first point refers to our observation that a two-pool model “fits the data better” than a one-pool model. As originally described by Lifson and McClintock (14), an assumption underlying the DLW method is that the isotopically labeled water (²H and ¹⁸O), after being introduced into the body, does not dilute beyond the body water pool. Since the dilution space of ²H is typically 1–4% larger than the dilution space of ¹⁸O and both are larger than the total body water pool when assessed by desiccation, this assumption is apparently not met, and other dilution pools and/or routes of elimination are thus in play. Therefore, one would expect that a two-pool model equation is a priori superior to a one-pool model, knowing that a two-pool model weighs the magnitudes of two separate routes of elimination in the final rCO₂ estimation rather than one route of elimination. In small animals (<1 kg), however, researchers tend to find that a one-pool model “fits the data better” than a two-pool model, although the differences are relatively small (19, 20) or nonexistent (2, 6). The answer to why differences are found relative to expectations remains elusive to date. If the underpinnings of this “rule of thumb” are not clear in the first place, it is of course quite impossible for us to give an answer as to why we find conditions in which the two-pool model approach is shown to be least discrepant in

estimating rCO₂ in our study (8). Rather than acknowledging the fact that this “rule of thumb” is apparently not rock solid, Yamada et al. (25) point out that we “did not mention this, test, or even discuss possible reasons for this discrepancy” and suggest that our data are “specific to [our] own laboratory”. We contend that more validation studies aimed at the heart of the matter should be done to clarify mechanisms underlying inconsistencies in the first place (20).

Second, the suited approach to validate rCO₂ estimated by the DLW method is to compare it with levels obtained by gas exchange measurements in an IC setting. We strongly oppose the view of Yamada et al. (25) that our IC “reference method is questionable” and that our IC data “are so discrepant from the rest of the literature.” We performed IC measurements exactly according to the methodology reported by Visser et al. (22), and that study was cited by us in our debated report (8). Specifically, the mass-flow controllers were calibrated with a soap foam flow meter (Bubble-O-Meter, La Verne, CA) before and after the trials, showing little variation over time (i.e., <1%). The infrared respiration gas analyzer was calibrated daily with two certified gas standards (AGA Gas, Amsterdam, The Netherlands), spanning the observed CO₂ gas concentrations between 0 and 0.5%. Daily adjustments of the span of the CO₂ gas analyzer were very small and were typically <1% of the certified CO₂ concentration. Therefore, we estimate the maximum overall error of our gas respiration method to be ~2%. Comparable methodologies were applied for O₂ calibration. We did not perform the ethanol-burning procedure for calibration as proposed by Yamada et al. (25), which is commonly applied in human studies (24). Although such methodology has certain advantages, downscaling the calibration procedure of quantitative ethanol combustion to small animal IC equipment can be problematic (21).

We do agree with Yamada et al. (25) that the respiratory quotient (RQ) of the mice in our study is higher than would be expected based on the estimated food quotient (FQ). Elevated RQ levels are, among others, reported as a consequence of exercise (10), lipogenesis (1), and stress (23), and these conditions are not included in estimation of the FQ. Particularly the latter two may have been relevant for our study. First, the frequent tail blood sampling procedure probably induced an increased level of discomfort in the mice, which subsequently increased RQ in all diet groups. Increased de novo lipogenesis would be expected to occur particularly in mice fed a HFS diet (i.e., the RQ for the conversion of glucose to fat is 5.55, whereas the RQs for oxidation of fat and glucose are 0.7 and 1, respectively), which would increase RQ further in this diet group above the FQ.

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Third, Yamada et al. (25) raise the issue that we believe a primary mechanism underlying our observed effect is increased de novo lipogenesis. This is a misconception; in the DISCUSSION of our article (8), we do not mention lipogenesis at all but instead bring up other possibilities like increased ketone body formation in the HFS-fed mice (4, 5), changes in gut microbiota (11, 15), or intracellular vs. extracellular discriminative mechanism (12, 13). These factors all need to be investigated further on their relative contributions to errors in $r\text{CO}_2$ assessment by DLW and, as correctly argued by Yamada et al. (25), possibly in IC too. This is to say that we do not dismiss the possibility that increased de novo lipogenesis could be a mechanism that may affect N_D and $r\text{CO}_2$ because of incorporation into C-H bonds (18), in addition to the presence of labile hydrogens, as suggested by Matthews and Gilker (16). However, these effects would force N_D and $r\text{CO}_2$ in the opposite direction. In fact, studies of Ravussin et al. (17) and Haggarty et al. (9) have previously shown underestimation of $r\text{CO}_2$ by the DLW method compared with IC, presumably as a result of increased lipogenesis. Unfortunately, a focus on the role of macronutrient composition of the diet is missing in these studies.

In our study, differences between dilution spaces N_D and N_O were observed particularly in the LF condition and became smaller in the mice fed the HFS diet, an effect that was slightly more evident in the HFS-fed mice that were obesity prone than those that were obesity resistant. We thank Yamada et al. (25) for pointing out a textual displacement in our article (8) on the ranges over which N_D/N_O varied using the plateau and intercept method. The correct reporting should have been that N_D/N_O ranged from 0.99 to 1.04 for the intercept method and from 1.02 to 1.07 for the plateau method. We understand that our data are at odds with the unpublished observations of Yamada et al. (25), apparently finding an increase in N_D/N_O as a result of fat feeding in rabbits, and that a single pool would fit the data better (according to theoretical considerations, without specifying what these exactly are). We are very interested in these data and hope to find them published soon. Yamada et al. (25) also criticized the use of our memory correction model (7), stating that “such adjustments can be subject to artifacts by changes in the performance of the system when sample matrixes are those other than water.” We have difficulties seeing the relevance of this remark; our system is fed exclusively with water samples, as all samples have been distilled on fore-hand. Moreover, our memory correction model rather removes artifacts that are due to (residual) memory effects, such as memory differences between hydrogen and oxygen that go unnoticed in other “brute force” memory correction schemes.

Fourth, despite the fact that we found inconsistencies in $r\text{CO}_2$ assessed by DLW and IC in mice fed the HFS diet, these discrepancies were not reflected in major deviations (between 2 and 5%) in the body water pool assessed by 4-h desiccation at 103°C vs. ^{18}O dilution (at least when 2- and 3-h initials in the intercept approach were considered). The desiccation method that we applied is a European standardized protocol, ISO 6496-1983[E], and has been certified to obtain dry mass in biological substances. We have carefully checked and approved this method in our laboratory for the purpose of assessing lean dry mass in mice according to the methodologies reported by Crum et al. (3). Although we were not able to compare both techniques to assess body water content in LF-fed mice (due to an accident with one of our freezers, these carcasses were lost), an overestimation of body water content

by ^{18}O dilution between 2 and 5% relative to desiccation in the HFS-fed mice is in our view comforting.

Apart from our debated study (8) and the unpublished findings that were mentioned in the commentary by Yamada et al. (25), to the best of our knowledge there are no reports in literature that have specifically addressed dietary effects on the validity of the DLW method in assessing $r\text{CO}_2$. In conclusion, we reemphasize that more research should be done to dissociate diet effects from obesity effects on the validity of the DLW method. This may be particularly relevant for biomedical and nutrition research.

AUTHOR CONTRIBUTIONS

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

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