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Validation of the DLW method in Japanese quail at different water fluxes using laser and IRMS

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Van Trigt, R., E. R. T. Kerstel, R. E. M. Neubert, H. A. J. Meijer, M. McLean, and G. H. Visser. Validation of the DLW method in Japanese quail at different water fluxes using laser and IRMS. J Appl Physiol 93: 2147–2154, 2002—In Japanese quail (Coturnix c. japonica; n = 9), the doubly labeled water (DLW) method (2H, 18O) for estimation of CO2 production (l/day) was validated. To evaluate its sensitivity to water efflux levels (rH2Oe; g/day) and to assumptions of fractional evaporative water loss (x; dimensionless), animals were repeatedly fed a dry pellet diet (average rH2Oe of 34.8 g/day) or a wet mash diet (95.8 g/day). We simultaneously compared the novel infrared laser spectrometry (LS) with isotope ratio mass spectrometry. At low rH2Oe, calculated CO2 production rate exhibited little sensitivity to assumptions concerning x, with the best fit being found at 0.51, and only little error was made employing an x value of 0.25. In contrast, at high rH2Oe, sensitivities were much higher with the best fit at x = 0.32. Conclusions derived from isotope ratio mass spectrometry and LS were similar, proving the usefulness of LS. Within a threefold range of rH2Oe, little error in the DLW method is made when assuming one single x value of 0.25 (recommended by Speakman JR, Doubly Labelled Water. Theory and Practice. London: Chapman & Hall, 1997), indicating its robustness in comparative studies.

isotope ratio mass spectrometry; laser spectrometry; doubly labeled water; energy expenditure; water flux

THE DOUBLY LABELED WATER (DLW) method has frequently been used for measuring the rate of CO2 production (rCO2) in free-living animals and humans and therewith their levels of energy expenditure (5, 6, 8, 12). Its usage is based on the measurement of the turnover rates of both 2H and 18O. It is hereby assumed that, after administration of a dose of 2H- and 18O-enriched water, 2H leaves from the body water pool exclusively as water, and 18O both as water and CO2 gas. Consequently, the difference between 18O and 2H turnover rates is proportional to the CO2 production. However, because of mass differences between 1H and 2H, as well as 16O and 18O, heavy isotopes leave the body water pool less readily in gaseous molecules such as water vapor and CO2 gas (fractionation effects).

These fractionation effects differ between isotopes within the same molecule (e.g., 2H and 18O in the water molecule; Ref. 6) and also for the same isotope in different molecules (e.g., 18O in water or CO2; Ref. 6). If no corrections are made for different fractionation processes, the calculated levels of CO2 production will be systematically too high. To account for this, some specific assumptions must be made for the fractions of water lost as liquid (not fractionated) and as vapor [fractionated evaporative water loss (x; dimensionless); Refs. 3, 12]. Originally, the x value was taken as 0.5, as estimated from small mammals under laboratory conditions, but this value has also been applied to free-living animals with all sorts of diets (6, 12). However, after having completed a more detailed analysis on water fluxes and evaporative water losses, Speakman (12) proposed the use of an x value of 0.25 for free-living animals.

Speakman (12) lists 22 validation studies of the DLW method for mammals and 18 for birds. In all studies, animals were housed in small cages, at thermoneutrality, and were fed a standard diet. Because birds in the field typically exhibit higher levels of energy expenditure, and thus higher levels of food and water intake, their water flux levels tend to be almost 60% higher than in the laboratory (10). Therefore, it is questionable whether the results of the laboratory-based validation experiments are directly applicable to free-living conditions. Moreover, animals of some species tend to have diets with large differences in water content during their annual cycle, resulting in large seasonal variations in water fluxes. For example, Red Knots (Calidris canutus) feeding on insects during the reproductive stage exhibit water fluxes of ~80 g/day; while feeding on bivalves during the migratory and wintering stages, these levels can reach values up to 600 g/day (16). Therefore, it is possible that the application of one specific x value for these birds throughout the annual cycle is not valid. An erroneous estimation for x will affect the overall accuracy of the DLW method by creating a systematic bias for the calculated levels of...
CO₂ production (12), potentially complicating the application of the DLW method in comparative studies. At high water fluxes (relative to the level of CO₂ production), there is little divergence between the elimination curves of both isotopes (11), resulting in a high sensitivity to analytic errors and thus in a reduction of the precision of the method (12). Therefore, especially if the DLW method is to be applied in animals at high water fluxes, a continuous need exists for improvement of analytic methods. The traditional way of determining isotope ratios in body water is through equilibration with CO₂ and conversion into H₂ gas for ¹⁸O and ²H, respectively, and subsequent measurement with dual-inlet isotope ratio mass spectrometry (IRMS). The analytic errors of the method are significant, especially for ²H (20). Recently, we developed a novel laser spectrometric (LS) method suitable for biomedical applications that has a number of advantages over the traditional techniques; among these are an enhanced precision of ²H measurement and a higher rate of sample analysis (14). The LS method is based on direct infrared laser absorption spectrometry of a water sample in the vapor phase, enabling a measurement of isotope ratios without performing any sample preparation steps.

To investigate the sensitivity of the DLW method at assumptions concerning x, a validation study was performed in Japanese quail (Coturnix c. japonica) against direct respiration gas analysis. This technique is very straightforward, can be performed with high accuracy, and does not rely on assumptions. As such, we apply the outcome of measurements using this technique as validation for our DLW experiments (in most validation studies referred to as “golden standard,” e.g., Ref. 12). To manipulate water flux rates within individuals, birds were fed either a standard pellet diet (resulting in a “normal” water flux for a laboratory animal) or the same standard diet but mixed with water to yield a wet mash diet with ~80% water (potentially resulting in a “high” water flux). Additionally, to explore the advantages of the newly developed LS with its higher precision for ²H measurements, LS-based results were compared with those derived from classic IRMS analysis.

METHODS

Animals and Housing

For the validation experiment, we used nine male Japanese quails of a fast-growing strain (broiler) between 10 and 15 wk of age. Before the validation measurements, birds were individually housed at 20°C in wooden keeping cages (length × width × height: 67 × 39 × 44 cm) and had access to drinking water ad libitum. Food, also available ad libitum, consisted of either a dry pellet diet (henceforth referred to as the “dry” diet) containing 27.7% (wt/wt) crude protein with a gross energy content of 17 kJ/g (1) or a wet mash diet (“wet” diet) using the same type of pellets dissolved in drinking water (mixing ratio of 1:4 wt/wt). The light-dark cycle was 16:8 h, with lights on at 0800. In all cases, birds were allowed to adjust to a particular diet for at least 1 wk. To avoid any bias, in five birds the validation was performed first when they were fed the dry diet and the wet diet thereafter. In the other four birds, we first performed the validation with the wet diet and the dry diet thereafter.

Experimental Procedures

Each bird was intraperitoneally injected with a dose water (with 45.2% ²H and 46.1% ¹⁸O) of ~3 mg/g body mass by using a sterile needle. Its exact quantity was determined by weighing the syringe before and after the administration on a Sartorius BP121S balance to the nearest 0.1 mg. After an equilibration period of 60 min (12, 15), the bird was weighed on a Sartorius QT6100 balance to the nearest 0.1 g. Subsequently, a blood sample of ~0.4 ml was taken from the bird after puncturing the brachial vein with a sterile needle (henceforth referred to as the “initial” blood sample). Samples were always stored at 4°C in a 1-ml glass tube until further analysis (see below). Immediately thereafter, the bird was individually placed in a respiration chamber (length × width × height: 35 × 25 × 25 cm), and the lid was closed. The respiration chamber was connected to a controlled airflow unit with a ventilation rate of 90 l/h, and it was placed in a climatized room with the same light-dark cycle as before. The temperature within the respiration chamber was kept between 15 and 16°C. The respiration chamber contained a metal grid above a 1.5-cm layer of paraffin oil. During the measurement, the bird had free access to water and food. Because of this setup, we were unable to measure evaporative water losses during the experiments. Twenty-four hours after the initial blood sample was taken, the lid was removed from the respiration chamber, the bird was reweighed, and another blood sample was taken from the vein of the opposite wing (henceforth referred to as “final” blood sample). To minimize interference of the sampling procedure with the animals’ behavior during the validation experiments, we refrained from taking an individual specific blood sample immediately before the injection (the “background” sample). Pilot experiments had revealed that an intensive sampling procedure negatively interfered with the animal’s food and water consumption, particularly when fed the wet diet. Therefore, a blood sample was collected from only four animals 2 days before the validation.

Infrared Respiration Gas Analysis

The rCO₂ values were measured in an open airflow system, as previously described (15, 17). In brief, respiration airflow, which was adjusted at 90 l/h, was controlled by a calibrated Brooks 5850 E mass-flow controller, to obtain an absolute difference in CO₂ concentration between inlet and outlet air of ~0.5%. These concentrations were determined every 2 min for each measurement with an infrared CO₂ gas analyzer (Leybold Heraeus BIONS-IR). The rCO₂ was calculated as the difference between the CO₂ fractions of the inlet and outlet air times the flow rate. Unfortunately, we failed to downscale the calibration procedure of quantitative ethanol combustion, as frequently used in validation studies of humans (18). Because of the high-energy content of the ethanol, with ventilation rates of 90 l/h (as employed during the validation study, being governed by the birds’ rCO₂), even the lowest possible combustion rates resulted in CO₂ concentrations of the “respiration” air considerably above the upper detection limit of 1%. Therefore, ethanol combustion cannot be used to mimic CO₂ production levels of small animals at low-ventilation rates. Alternatively, we used the following procedures to calibrate our equipment (see also Refs. 15 and 17). Mass-flow controllers were calibrated with a soap foam flowmeter (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), spanning the observed CO₂ gas concentrations between 0 and 0.5%. CO₂ concentrations of these reference gasses were gravimetrically verified during an interlaboratory comparison (15). 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%.

Isotope Analysis

First, each blood sample was distilled in a vacuum line, and the distillate was cryogenically trapped in a 1-ml glass tube. In preliminary studies, it had been verified that this distillation procedure did not cause a change in the isotope enrichment level. Second, part of the distillate was flame sealed in six glass microcapillary tubes (10–15 μl each), as dictated by the IRMS analytical procedure. The remainder of the distillate was used for LS analysis. As internal standards, a dilution series of the DLW injection mixture with natural tap water of known isotopic composition was used. These were analyzed in the same batches as the distilled blood samples (see Ref. 14) and had been calibrated against a range of International Atomic Energy Agency standards.

IRMS. The capillaries were mechanically broken inside an evacuated system and frozen into a glass vial (for more details, see Ref. 15). In brief, we used the Epstein-Mayeda method to equilibrate the water at 25.0°C with a known amount (2.00 ml) of added CO₂ gas of known isotopic composition (2). After an equilibration period of at least 48 h, CO₂ was cryogenically trapped in a glass vial for measurement with the IRMS. The remaining water was led over a uranium tube. In preliminary studies, it had been verified that this procedure did not cause a change in the isotope enrichment level. After an equilibration period of at least 48 h, CO₂ was cryogenically trapped in a glass vial for measurement with the IRMS. The remaining water was led over a uranium tube. In preliminary studies, it had been verified that this procedure did not cause a change in the isotope enrichment level.

Calculations in the DLW Method

Isotope abundance ratios. The average ¹⁸Ο/¹⁶Ο and ²H/²H isotope abundance ratios of each sample (Rₛ; dimensionless) were expressed as relative deviations from the international standard Vienna standard mean ocean water (VSMOW)

\[ ^{\delta}S = (R_s/R_{VSMOW}) - 1 \quad (1) \]

and, thus

\[ ^{\delta}S = (R_s/R_{VSMOW}) \times (1 + ^{\delta}\delta) \quad (2) \]

where the superscript x is the mass of the rare isotope, \( R_{VSMOW} \) is the isotope abundance ratio of VSMOW, and \( ^{\delta}\delta \) is the isotope ratio of the sample (%). For further calculations, the absolute isotope concentrations of the samples were used. For example, for ¹⁸Ο (¹⁸C₂)

\[ ¹⁸C₂ = ¹⁸R_s/(1 + ¹⁸R_s) \quad (3) \]

where \( ¹⁸R_s \) is the \( R_s \) value for ¹⁸Ο. The concentrations were expressed in parts per million.

Amount of total body water. The amount of total body water (TBW; in g) for each individual animal can be determined following the principle of isotope dilution

\[ \text{TBW} = 18.02 \cdot Q_d \cdot (C_d - C_f)/(C_i - C_f) \quad (4) \]

(13), where \( Q_d \) is the individual-specific quantity of the dose (mol), \( C_d \) is the isotope concentration of the dose, \( C_i \) is the individual-specific isotope concentration of the initial blood sample, and \( C_f \) is the population-specific average background concentration. This method is often referred to as the plateau method (12) and can be applied for each administrated isotope.

Fractional turnover rates. For each isotope, the fractional turnover rate (k; 1/day) during the experiment can be calculated

\[ k = (1/t) \cdot \ln(C_i - C_f)/(C_i - C_d) \quad (5) \]

where \( t \) is the time interval between initial and final sample (days), and \( C_i \) is the isotope concentration of the final sample. The \( k \) values of ²H and ¹⁸Ο are referred to as \( k_2 \) and \( k_{18} \), respectively.

Water fluxes. Under steady-state conditions (i.e., the size of the body water pool remains constant during the measurement period), the water efflux (\( r_{H2O}; g/day \)) can, in first approximation without corrections for isotope fractionation, be calculated by

\[ r_{H2O} = 18.02 \cdot N \cdot k_2 \quad (6) \]

where \( N \) is the amount of body water (mol) determined from ¹⁸Ο dilution. This way of calculating TBW is considered to best reflect the correct value, because, for deuterium, a greater dilution space seems to exist: part of the dose is likely being incorporated in protein and fat (12). In this particular case, \( r_{H2O} \) equals the water influx level (\( r_{H2O}; g/day \)). The water flux can be manipulated by way of changing the diet of the animals.

As birds did not maintain a constant body mass during the validation measurements (see Table 1), \( r_{H2O} \) for each measurement was calculated by using Eqs. 5 and 6 of Visser et al. (16), which allow specific adjustments for \( x \) during non-steady-state conditions. Additionally, for each measurement, \( r_{H2O} \) was calculated by using Eq. 6 of Nagy and Costa (9).

CO₂ production. The \( r_{CO2} \) (mol/day) is, again in first approximation without corrections for isotope fractionation, given by

\[ r_{CO2} = (N/2) \cdot (k_{18} - k_2) \quad (7) \]

To correct for fractionation, three fractionation factors have already been defined by Löffon et al. (5). These take into account the evaporation of water for ²H (\( f_2; \) dimensionless) and ¹⁸Ο (\( f_2; \) dimensionless) and the CO₂-H₂O fractionation for ¹⁸Ο (\( f_3; \) dimensionless). To enable incorporation of these factors into Eqs. 6 and 7, the proportion of water lost as vapor
(fractionated; x; dimensionless) must be defined (6). After taking these fractionation effects into account, Eq. 6 can be rewritten as

\[ r_{\text{CO}_2} = \ln(N/2f_2) \cdot (k_{18} - k_2) - x \cdot [(f_2 - f_1)/(2f_3)] \cdot N \cdot k_2 \quad (8) \]

The numerical values of \( f_1 \) and \( f_2 \) are dependent on the exact pathways that are responsible for the evaporation of water: both equilibrium and kinetic isotope fractionation processes will contribute to the final values for \( f_1 \) and \( f_2 \). The assumed values of the equilibrium and kinetic fractionation for water evaporation can be obtained from literature (3, 12). For \( f_1 \) these are 0.941 and 0.9235, respectively, at 37°C, and for \( f_2 \), they are 0.9925 and 0.9760, respectively [following Speakman’s (12) notation]. It is hard to define exactly what fraction of the evaporating water is lost under which regime, and this fraction may even be temperature dependent (19). The most recent estimate for many small animals for the relative contribution of both evaporation processes is equilibrium/kinetic = 3:1 (3, 12). This relation is now widely used. For \( f_3 \), only equilibrium fractionation takes place, because CO2 is in constant equilibration with the water in the blood stream (fast equilibrium establishment due to the presence of the carbonic anhydrase) and in the lungs, before it can leave the body. The value of this fractionation factor is estimated to be 1.039 (3, 12). All values of the fractionation constants and their relative contributions are approximations, based on laboratory experiments.

Filling in the above-mentioned assumed values in Eq. 8 yields the following equation

\[ r_{\text{CO}_2} = \ln(N/2.078) \cdot (k_{18} - k_2) - x \cdot 0.0249 \cdot N \cdot k_2 \]  
\( (9) \)

Using Eq. 9, we calculated \( r_{\text{CO}_2} \) values based on IRMS (\( r_{\text{CO}_2,\text{IRMS}} \)) and LS (\( r_{\text{CO}_2,\text{LS}} \)) measurements. All calculated \( r_{\text{CO}_2} \) values were compared with the direct respiration gas analysis. The obtained results are expressed as the error against the direct respiration gas measurements in percent.

Assumptions concerning x. Assuming that the estimates for the fractionation factors and the employed ratio of equilibrium vs. kinetic isotope fluxes are correct, Eq. 9 can be applied for different assumptions concerning x. To circumvent the lack of knowledge on the individual-specific value for this parameter, it was originally taken as 0.5 for all diets, based on laboratory estimates of small mammals (6). Although this value has been widely used to estimate \( r_{\text{CO}_2} \) in free-living birds and mammals, a more detailed analysis suggested that a value of 0.25 was more appropriate because of the fact that water fluxes tend to be higher in free-living animals than in the laboratory (10, 12). To evaluate these specific assumptions in Japanese quails fed two different diets, for each bird \( r_{\text{CO}_2} \) was calculated from Eq. 9 at x levels of 0 (i.e., no evaporative water loss), 0.25, and 0.5. To refine the analysis of the importance of x on the errors of the DLW method, a more versatile approach was employed to calculate the best fit for x (error of the DLW method is zero relative to respiration gas analysis), following a recent sensitivity analysis carried out by Visser and Schekkerman (17).

RESULTS

Body Mass and Isotope Dilution Space

Average body masses (Table 1) of the Japanese quails were significantly higher when the animals were fed the dry diet than when fed the wet diet (261.4 and 246.9 g, respectively, paired t-test (two-tailed) \( t_8 = 4.19, P < 0.002 \)). However, TBW estimates (Table 1)
did not differ significantly between both diets, when calculated based on $^2\text{H}$ or $^{18}\text{O}$ dilution measured with IRMS ($P=0.29$ and 0.48, respectively) and when based on $^2\text{H}$ and $^{18}\text{O}$ measured with LS ($P=0.26$ and 0.49, respectively). In addition, changes in body mass during the measurements were significantly larger for birds fed the wet diet (7.4 g; SD=2.7 g) than when fed the dry diet (2.7 g; SD=4.0 g, paired $t$-test (two-tailed) $t_{8}=2.68, P<0.028$). This may be the result of a larger impact of the 1-h fasting period on body mass of birds when fed the wet diet (higher rate of defecation) then when fed the dry diet, resulting in lower body masses when the initial sample is taken.

To evaluate the effect of the analytic tool on the TBW estimates, $^2\text{H}$ isotope dilution space values were compared. It was found that, for both the dry and wet diet, values based on IRMS analysis statistically significantly exceeded those based on LS analyses ($P=0.002$ and 0.015, respectively), although this difference was very small (<1%). However, for the $^{18}\text{O}$ isotope dilution space values, it was found that they did not differ significantly for the two analytic tools employed (dry diet $P=0.08$, wet diet $P=0.19$).

To evaluate differences in dilution spaces between both isotopes, for IRMS-based values it was found that $^2\text{H}$ dilution spaces significantly exceeded those for $^{18}\text{O}$ by 3.0% on the average (dry diet $P=0.004$, wet diet $P<0.001$). For LS-based values for the dry diet, it was found that $^2\text{H}$ dilution spaces exceeded those for $^{18}\text{O}$ by 1.1%, but this was not statistically significant ($P=0.09$). In contrast, for the wet diet it was found that $^2\text{H}$ dilution spaces significantly exceeded those for $^{18}\text{O}$ by 2.8% on the average ($P<0.001$).

**Water Flux**

As a result of the manipulation of the diet, we were able to significantly change $r_{\text{H}_{2}\text{O}}$, by a factor of ~2.7 ($P<0.001$, Table 1). For the dry diet, values based on IRMS measurements were 34.8 and 35.3 g/day after assuming $x$ values of 0.25 and 0.50, respectively, proving little sensitivity of the calculated water flux to assumptions concerning $x$ (Table 1). Corresponding values for $r_{\text{H}_{2}\text{O}}$ were 36.4 (SD = 9.3) and 37.0 (SD = 9.5) g/day, respectively. For the wet diet, corresponding values for $r_{\text{H}_{2}\text{O}}$ were 96.0 and 97.5 g/day, respectively (Table 1), and for $r_{\text{H}_{2}\text{O}}$, values were 100.6 (SD = 13.7) and 102.1 (SD = 13.8) g/day, respectively. Only for the dry diet, calculated $r_{\text{H}_{2}\text{O}}$, values based on LS measurements were significantly lower than values based on IRMS measurements (for $x=0.25$, $P=0.034$, and for $x=0.5$, $P=0.040$), but the difference was <3%.

**CO$_2$ Production in Relation to x**

The $r_{\text{CO}_2}$ values, as measured with respiration gas analysis, are listed in Table 2. After we employed a paired $t$-test, it was found that the values did not differ significantly for both diets ($t_{8}=1.20, P=0.27$). Based on average $r_{\text{H}_{2}\text{O}}$, values (based on IRMS and LS analyses), water economy index (WEI; g H$_2$O/l CO$_2$) values were calculated for each measurement by dividing $r_{\text{H}_{2}\text{O}}$ by the daily $r_{\text{CO}_2}$ as assessed from infrared respiration gas analysis.
gas analysis (Table 2; Ref. 10). For the dry and wet diets, average WEI values were found to be 3.3 (SD = 0.5) and 8.5 (SD = 1.1) g H₂O/l (175%) CO₂, i.e., a 2.6-fold increase for the wet diet. To simplify the presentation of the results of the validation, rCO₂-IRMS and rCO₂-LS values are expressed as a relative deviation from the value obtained with the respiration gas analysis (Table 2).

For the dry diet, calculated rCO₂ values tended to be rather insensitive to assumptions concerning x (Table 2). For example, in the absence of evaporative water loss (x = 0), the average relative error of rCO₂-IRMS was 4.3%, whereas at x = 0.5, the average relative error was −0.7%. For this diet, at the three different assumed x levels, relative errors of the DLW method were lowest at x = 0.5 for rCO₂-IRMS and rCO₂-LS, amounted to −0.7 and 0.8%, respectively, and did not differ significantly from zero (P values: 0.81 and 0.76, respectively). At these assumed x levels, the standard deviations for these two methods were 7.9 and 6.9%, respectively, indicating similar precision levels. In addition, it was found that, when assuming x = 0.25, average errors did not differ significantly from zero for both methods (Table 2, P values: 0.53 and 0.21, respectively). For rCO₂-IRMS and rCO₂-LS, zero errors for the calculated mean rCO₂ were observed at x levels of 0.48 and 0.58, respectively, to yield an average value of 0.51. However, it has to be noted that, because of the low sensitivity of rCO₂ to assumptions concerning x for the dry diet, the application of an x value of 0.58 (as derived from rCO₂-LS) for the calculation of rCO₂-IRMS does not lead to an error significantly different from zero.

For the wet diet, calculated rCO₂ values are much more sensitive to assumptions concerning x. For example, at x = 0, the relative error based on the IRMS measurements was 8.5%, whereas at x = 0.5 its error was −4.3%. For this diet, lowest relative errors were observed at x = 0.25 for the rCO₂-IRMS and rCO₂-LS values, and the relative errors were on average 2.1 and 1.6%, respectively, but did not differ significantly from zero (P values: 0.32 and 0.51, respectively). Standard deviations for both methods were 5.6 and 6.6%, respectively, again suggesting that the precision of both analytic tools is comparable. In addition, it was found that, at an assumed x level of 0.5, there was a tendency that both methods underestimated the true rCO₂ by 4.3 and 4.8% for rCO₂-IRMS and rCO₂-LS, respectively, but this was not significant (Table 2, P values: 0.067 and 0.068), possibly because of the fact that the relative errors exhibited considerable variation. For these two estimates, zero error of calculated mean rCO₂ was obtained at x levels of 0.33 and 0.31, respectively, to yield an average value of 0.32.

To further statistically evaluate whether errors of rCO₂ are attributable to random analytic uncertainties, rCO₂-IRMS and rCO₂-LS values were averaged for each animal and diet. Subsequently, for each individual and diet, errors were calculated of these combined estimates relative to respiration gas analysis. For the dry diet, it was found that, for an x value of 0.25, average error was 2.5% (SD = 6.21%), and for a value of 0.5 the average error was 0.1% (SD = 6.40%). By comparison with the SD values for the separate analytic methods for the dry diet (Table 2), it can be calculated that the combined estimates are −14% more precise. Similarly, for the wet diet, it was found that the precision of the combined estimates was on average 28% better, indicating the higher sensitivity of the DLW method to analytic errors for the wet diet.

DISCUSSION

Water Flux in the Laboratory in Relation to Diet and Assumed x-levels

By manipulating the water content of the diet, in the Japanese quail, rH₂O increased significantly from 34.8 g/day when the animals were fed the dry diet (average value based on both analytic methods at x = 0.5) to 95.8 g/day for the wet diet (average value based on both methods at x = 0.25), i.e., an increase by 175% (Table 1). Additionally, WEI values increased from 3.3 to 8.5 g H₂O/l CO₂, i.e., an increase of 158%.

For the dry diet, the DLW method exhibited little sensitivity to assumptions concerning x, and, for the three levels evaluated (x = 0, 0.25, and 0.5), x = 0.5 was found to be the most appropriate. However, no significant error was made after assuming an x value of 0.25. For the wet diet, the DLW method appeared to be more sensitive to assumptions concerning x, with a best fit being found at a value of 0.25. The best fit of the DLW method for the dry and wet diets (i.e., overall error is zero) yielded x estimates of 0.51 and 0.32, respectively.

Water efflux can be partitioned in a route subject to fractionation (x, evaporative water loss) and another route not subject to fractionation (1 − x, mainly faecal and urinary water loss; Refs. 3, 6, 12). Although we have been unable to measure evaporative water losses of animals when they were fed the dry and wet diets, we are able to assess partitioning of water efflux using the calculated average rH₂O values (34.8 and 95.8 g/day, respectively) and the x values for which the average errors of the DLW method were zero (x values of 0.32 and 0.51, respectively). For the dry diet, evaporative and nonevaporative water losses were calculated to be 17.7 and 17.1 g/day, whereas for the wet diet corresponding values were calculated to be 30.7 and 65.1 g/day, respectively. Interestingly, calculated evaporative and nonevaporative water fluxes tended to be higher for the wet diet, but the change was smaller for the former route (73% increase) than for the latter (282%).

Comparison Between Observed Water Fluxes to Laboratory- and Field-based Allometric Predictions

A comprehensive review of literature data on water influx rates revealed that, for free-living birds, levels tend to be higher by, on average, almost 60% than for birds under laboratory conditions (10). In some aquatic birds, however, such as shorebirds and ducks, water
fluxes in the field tend to be even more elevated. For example, in captive Tufted ducks (Aythia fuligula), water fluxes were on average 172 g/day, but they were on average 827 g/day under free-living conditions (J. J. De Leeuw and G. H. Visser, unpublished observations). A similar range of values has been observed in the Red Knot (16).

To evaluate observed average $r_{H2O}$ levels for the Japanese quail fed the dry and wet diets, they were compared with allometric predictions based on existing data for birds under laboratory conditions (10). It was found that, for the dry and wet diets, $r_{H2O}$ value fluxes were, on average, 11.9% below and 152% above prediction, respectively. Similarly, based on field-based predictions, it was found that, for these diets, $r_{H2O}$ values were on average 43% below and 60% above prediction, respectively. Thus observed $r_{H2O}$ values fell in the range as observed in captive and free-living birds.

**Sensitivity of Calculated $r_{CO2}$ to Assumptions Concerning $x$: a Recommendation for the Application of the DLW Method in Comparative Studies on Free-living Animals**

For the wet diet, $r_{CO2}$ values exhibited a much greater sensitivity to assumptions concerning $x$ than for the dry diet. For example, for the wet diet, the relative error of $r_{CO2,IRMS}$ changed from 8.5% at an assumed $x$ value of 0 to −4.3% for an assumed $x$ value of 0.5% (Table 2). For both analytic tools employed, the best fit for the wet diet was obtained at an $x$ value of 0.32. For the dry diet, at both assumed $x$ levels, the average errors were 4.3 and −0.7%, respectively. A similar pattern was observed for $r_{CO2,LS}$ values. For both analytic tools employed, the best fit for the dry diet was obtained at an $x$ value of 0.51.

The uncertainty with respect to $x$ in free-living animals has been subject to debate for many decades (6, 8, 12, 15). Presently, since the application of the DLW method in small animals, groups of scientists have favored three different assumptions: 1) fractionation due to evaporation does not occur (i.e., $x = 0$; Refs. 6 (Eq. 6) and 7); 2) $x = 0.25$ (Ref. 12, Eq. 7.17); and 3) $x = 0.5$ (Ref. 6, Eq. 35). This uncertainty strongly complicates the application of the DLW in comparative studies, as the calculated $r_{CO2}$ is negatively correlated to the assumed level of $x$. Given the large differences in water fluxes between captive and free-living animals, it is questionable whether these issues can be adequately resolved in laboratory-based validation studies.

As we have shown in Table 2, the sensitivity of $r_{CO2}$ to assumptions concerning $x$ tends to be a function of the animal’s water flux. More specifically, Visser and Schekkerman (17) and Visser et al. (15) demonstrated that this sensitivity is a function of the animal’s water flux per unit of CO$_2$ production (10). Their findings (15, 17) were confirmed in our study. When animals were fed the dry diet (WEI = 3.3 g H$_2$O/l CO$_2$), assumptions concerning $x$ exhibited rather little effect on the calculated $r_{CO2}$, whereas, when they were fed the high diet (WEI = 8.5 g H$_2$O/l CO$_2$), this effect was much larger (Table 2). At high water fluxes per unit of CO$_2$ production (i.e., in animals fed the wet diet), there is relatively little difference between $^2$H and $^{18}$O turnover rates, and any small change in the assumed $x$ will have a significant impact on the calculated $r_{CO2}$ value. Conversely, at low water fluxes per unit of CO$_2$ production (i.e., in animals fed the dry diet), this sensitivity is much less. Given these uncertainties, we have shown that the overall error of the DLW method for the dry and wet diets does not differ significantly from zero at an assumed $x$ level of 0.25. Based on this finding, of the three $x$ values presently used, we tentatively propose usage of $x = 0.25$ for calculation of $r_{CO2}$ in comparative studies in free-living animals. However, it would be worthwhile to execute more validation studies for animal species exhibiting high water fluxes, enabling a species- and diet-specific $x$ value. It has to be reemphasized that these $x$ values strongly depend on the values taken for the fractionation constants (Eq. 8). Clearly, more research is needed to provide better estimates for these constants and their relationships to body temperatures (3, 12, 19).

**Perspectives: LS as an Analytic Tool for DLW Studies**

For DLW applications with stable isotopes, dual-inlet IRMS has traditionally been used as an analytic tool to yield the highest overall accuracy and precision of the method (20). IRMS measurement requires the conversion of the sample of the body water pool to gasses of small molecules such as H$_2$ and CO$_2$. This conversion is not without problems, especially the reduction of the water molecule to yield H$_2$ gas, potentially affecting the precision and accuracy of the DLW method. As we have shown above, this is especially the case in animals exhibiting high water fluxes per unit of CO$_2$ production. Therefore, there is a continuous need for improvement of the analytic tools. In the framework of a larger research project (4), we now have evaluated the novel LS method as an analytic tool. The analyses have revealed (Tables 1 and 2) that both accuracy and precision of LS are at about the same level as observed in traditional IRMS. However, it has to be mentioned here that our present application of the IRMS as an analytic tool is the product of a 45-yr development, whereas this is our first application of the LS. In combination with a higher sample throughput of LS compared with IRMS (4), we firmly believe that LS analysis will eventually outclass IRMS analysis. Moreover, we are presently evaluating another advantage of LS: its ability to measure $^{17}$O enrichments along with $^2$H and $^{18}$O. This would enable us to continue the pioneering work of Haggarty et al. (3) to further develop the promising triply labeled water method, but with stable isotopes. This potentially has the advantage of calculating $r_{CO2}$ based on $^2$H and $^{18}$O turnover rates, as well as on $^3$H and $^{17}$O, a possibility that has been explored in the literature with radioactive (3) but not with stable isotopes.
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