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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; 10.1152/japplphysiol.01134.2001.—In Japanese quail (*Coturnix c. japonica*; $n = 9$), the doubly labeled water (DLW) method (^{2}H, ^{18}O) for estimation of CO_2 production (l/day) was validated. To evaluate its sensitivity to water efflux levels ($r_{H_2O_e}$; g/day) and to assumptions of fractional evaporative water loss (x; dimensionless), animals were repeatedly fed a dry pellet diet (average $r_{H_2O_e}$ 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 (IRMS). At low $r_{H_2O_e}$, calculated CO_2 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 $r_{H_2O_e}$, 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 $r_{H_2O_e}$, 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 CO_2 production (r_{CO_2}) 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 ^{2}H and ^{18}O. It is hereby assumed that, after administration of a dose of ^{2}H- and ^{18}O-enriched water, ^{2}H leaves from the body water pool exclusively as water, and ^{18}O both as water and CO_2 gas. Consequently, the difference between ^{18}O and ^{2}H turnover rates is proportional to the CO_2 production. However, because of mass differences between ^{1}H and ^{2}H, as well as ^{16}O and ^{18}O, heavy isotopes leave the body water pool less readily in gaseous molecules such as water vapor and CO_2 gas (fractionation effects).

These fractionation effects differ between isotopes within the same molecule (e.g., ^{2}H and ^{18}O in the water molecule; Ref. 6) and also for the same isotope in different molecules (e.g., ^{18}O in water or CO_2; Ref. 6). If no corrections are made for different fractionation processes, the calculated levels of CO_2 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

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CO_2 production (12), potentially complicating the application of the DLW method in comparative studies.

At high water fluxes (relative to the level of CO_2 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 CO_2 and conversion into H_2 gas for ^{18}O 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 to 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% ^{18}O) 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 r_{CO_2} 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 CO_2 concentration between inlet and outlet air of ~0.5%. These concentrations were determined every 2 min for each measurement with an infrared CO_2 gas analyzer (Leybold Heraeus BIONS-IR). The r_{CO_2} was calculated as the difference between the CO_2 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' r_{CO_2}), even the lowest possible combustion rates resulted in CO_2 concentrations of the "respiration" air considerably above the upper detection limit of 1%. Therefore, ethanol combustion cannot be used to mimic CO_2 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 CO_2 gas concentrations between 0 and 0.5%. CO_2 concentrations of these reference gasses were gravimetrically verified during an interlaboratory comparison (15). Daily adjustments of the span of the CO_2 gas analyzer were very small and were typically <1% of the certified CO_2 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 analytic 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 CO_2 gas of known isotopic composition (2). After an equilibration period of at least 48 h, CO_2 was cryogenically trapped in a glass vial for measurement with the IRMS. The remaining water was led over a uranium oven at 800°C to produce uranium oxide and H_2 gas, which was cryogenically trapped in a glass vial with active charcoal. The CO_2 and H_2 were measured on a VG-SIRA 10 and VG-SIRA 9 to obtain the $^{18}O/^{16}O$ and $^2H/^1H$ isotope abundance ratios, respectively, of the original blood sample. All samples were prepared in quadruplicate, and values were averaged. A correction for cross-contamination (7) was applied. Then, all isotope ratio measurements were calibrated as recommended by the International Atomic Energy Agency (20) against the gravimetrically mixed internal standards. Because relative uncertainties in the weighing procedure are much smaller than the measurement precision with IRMS, the internal standards are considered as absolute (see also Ref. 14).

LS. The same distilled water samples and standards were used (for more details, see Ref. 14). In brief, for each measurement, 10.0 μl of liquid water were injected into the gas cell through a rubber septum. After evaporation of the water sample, 12 absorption spectra of the sample and working standard were recorded, and a mean $^2H/^1H$ and $^{18}O/^{16}O$ isotope abundance ratio was then calculated from these spectra. For each sample, this procedure was repeated five times, and values were averaged. Calibration was done against the same internal standards. We have recently made a comparison of the accuracy of IRMS and LS; a more detailed description can be found in Ref. 14. Background samples were only measured with IRMS because of their too small sizes.

Calculations in the DLW Method

Isotope abundance ratios. The average $^{18}O/^{16}O$ and $^2H/^1H$ isotope abundance ratios of each sample (^xR_s; dimensionless) were expressed as relative deviations from the international standard Vienna standard mean ocean water (VSMOW)

$$^x\delta_s = (^xR_s/^xR_{VSMOW}) - 1 \qquad (1)$$

and, thus

$$^xR_s = {}^xR_{VSMOW} \cdot (1 + {}^x\delta_s) \qquad (2)$$

where the superscript x is the mass of the rare isotope, $^xR_{VSMOW}$ is the isotope abundance ratio of VSMOW, and $^x\delta_s$ is the isotope ratio of the sample (‰). For further calculations, the absolute isotope concentrations of the samples were used. For example, for ^{18}O ($^{18}C_s$)

$$^{18}C_s = {}^{18}R_s/(1 + {}^{18}R_s) \qquad (3)$$

where $^{18}R_s$ is the ^xR_s value for ^{18}O. 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

$$TBW = 18.02 \cdot Q_d \cdot (C_d - C_i)/(C_i - C_b) \qquad (4)$$

(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_b 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

$$k = (1/t) \cdot \ln([C_i - C_b]/[C_f - C_b]) \qquad (5)$$

where t is the time interval between initial and final sample (days), and C_f is the isotope concentration of the final sample. The k values of ^2H and ^{18}O 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 ($r_{H_2O_e}$; g/day) can, in first approximation without corrections for isotope fractionation, be calculated by

$$r_{H_2O_e} = 18.02 \cdot N \cdot k_2 \qquad (6)$$

where N is the amount of body water (mol) determined from ^{18}O 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, $r_{H_2O_e}$ equals the water influx level ($r_{H_2O_i}$, 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), $r_{H_2O_e}$ 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, $r_{H_2O_i}$ was calculated by using *Eq. 6* of Nagy and Costa (9).

CO_2 production. The r_{CO_2} (mol/day) is, again in first approximation without corrections for isotope fractionation, given by

$$r_{CO_2} = (N/2) \cdot (k_{18} - k_2) \qquad (7)$$

To correct for fractionation, three fractionation factors have already been defined by Lifson et al. (5). These take into account the evaporation of water for ^2H (f_1; dimensionless) and ^{18}O (f_2; dimensionless) and the CO_2-H_2O fractionation for ^{18}O (f_3; dimensionless). To enable incorporation of these factors into *Eqs. 6* and *7*, the proportion of water lost as vapor

Table 1. *Individual-specific body masses, calculated amounts of body water based on IRMS and LS analyses, and calculated water efflux rates based on IRMS and LS analyses, assuming x values of 0.25 and 0.5*

Animal No.	Mass, g	TBW IRMS, g		TBW LS, g		$r_{H_2O_e}$ IRMS, g/day		$r_{H_2O_e}$ LS, g/day	
		^{18}O	^2H	^{18}O	^2H	$x = 0.25$	$x = 0.5$	$x = 0.25$	$x = 0.5$
				Dry diet					
1	204.5(2.0)	123.3	127.5	124.0	127.9	31.2	31.6	29.6	30.1
2	248.7(4.0)	151.0	157.2	152.1	155.2	23.3	23.6	22.7	23.1
3	240.7(4.7)	148.0	153.0	148.7	153.3	41.9	42.6	40.1	40.7
4	260.9(2.7)	141.4	148.3	141.9	146.4	31.8	32.3	32.8	33.3
5	322.6(2.8)	205.1	209.4	204.3	206.2	47.9	48.6	48.0	48.8
6	292.9(0.5)	138.2	135.3	138.5	133.2	21.7	22.1	21.0	21.3
7	277.4(6.4)	143.1	147.5	143.4	145.4	47.1	47.8	46.4	47.2
8	269.1(7.6)	155.0	158.0	155.9	156.0	38.1	38.7	34.6	35.1
9	236.2(6.3)	139.2	139.9	138.6	138.1	29.9	30.4	29.6	30.1
Average	261.4(2.7)	149.3	152.9	149.7	151.3	34.8	35.3	33.9	34.4
SD	34.5(4.0)	22.8	23.5	22.5	22.8	9.6	9.7	9.5	9.7
				Wet diet					
1	200.9(8.8)	146.3	152.3	146.3	150.0	116.5	118.3	116.2	118.0
2	235.1(4.6)	144.2	148.9	143.7	147.0	93.1	94.1	91.8	93.2
3	228.0(4.4)	151.8	158.8	151.5	155.7	112.9	114.7	112.9	114.6
4	244.5(8.2)	129.4	135.9	129.8	133.0	77.8	79.1	78.2	79.4
5	285.9(12.1)	186.5	192.6	186.4	192.5	92.3	94.4	92.6	94.1
6	290.9(3.9)	134.5	139.3	135.0	138.7	81.0	82.3	81.5	82.8
7	255.2(9.3)	151.8	156.2	152.4	157.4	99.1	100.7	96.2	97.7
8	260.3(8.8)	154.3	162.1	155.9	160.4	100.2	101.8	102.2	103.8
9	221.1(6.5)	147.8	152.2	147.4	152.0	90.1	91.5	88.6	90.0
Average	246.9(7.4)	149.6	155.4	149.8	154.1	95.9	97.4	95.6	97.1
SD	29.5(2.7)	16.1	16.4	16.1	16.9	13.0	13.2	12.9	13.1

Birds were fed either a dry or a wet diet. Mass values in parentheses are individual changes in body mass during the measurement period. TBW, total body water; IRMS, isotope ratio mass spectrometry; LS, laser spectrometry; $r_{H_2O_e}$, water efflux rate; *x,* fractional evaporative water loss.

(fractionated; *x*; dimensionless) must be defined (6). After taking these fractionation effects into account, *Eq. 6* can be rewritten as

$$r_{CO_2} = (N/2f_3) \cdot (k_{18} - k_2) - x \cdot [(f_2 - f_1)/(2f_3)] \cdot N \cdot k_2 \quad (8)$$

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 ^2H (f_1), these are 0.941 and 0.9235, respectively, at 37°C, and, for ^{18}O (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 CO_2 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

$$r_{CO_2} = (N/2.078) \cdot (k_{18} - k_2) - x \cdot 0.0249 \cdot N \cdot k_2 \quad (9)$$

Using *Eq. 9*, we calculated r_{CO_2} values based on IRMS ($r_{CO_2\text{-}IRMS}$) and LS ($r_{CO_2\text{-}LS}$) measurements. All calculated

r_{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_{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_{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)

Table 2. *Relative errors in CO_2 production rates of Japanese quail as determined with the IRMS and LS methods relative to the direct respiration gas analysis*

Animal No.	$r_{CO_2\text{-IRGA}}$, l/day	IRMS Error, %			LS Error, %		
		$x = 0$	$x = 0.25$	$x = 0.50$	$x = 0$	$x = 0.25$	$x = 0.50$
			Dry diet				
1	9.2	−1.3	−4.0	−6.8	6.4	3.9	1.3
2	9.5	3.4	1.3	−0.7	14.7	12.7	10.7
3	11.6	8.3	5.3	2.4	6.8	4.0	1.1
4	11.8	15.8	13.6	11.4	3.7	1.4	−0.8
5	14.9	6.4	3.8	1.3	5.1	2.6	0.0
6	9.4	5.1	3.3	1.6	4.5	2.8	1.0
7	12.2	−1.8	−4.5	−7.2	−5.6	−8.3	−10.9
8	10.7	−8.7	−11.8	−14.8	−0.4	−3.1	−5.9
9	10.0	11.8	9.2	6.6	15.6	13.1	10.6
Average	11.0	4.3	1.8	−0.7	5.7*	3.2	0.8
SD	1.8	7.5	7.7	7.9	6.6	6.7	6.9
			Wet diet				
1	11.2	9.5	2.4	−4.7	9.8	2.7	−4.4
2	10.7	10.1	3.3	−3.6	11.3	4.5	−2.3
3	14.1	15.2	8.9	2.6	8.1	1.9	−4.4
4	11.5	9.1	3.7	−1.8	2.3	−3.2	−8.7
5	12.4	10.7	4.5	−1.7	6.6	0.4	−5.8
6	9.9	13.2	6.9	0.5	14.1	7.8	1.3
7	11.5	6.7	−0.3	−7.3	17.5	10.7	3.8
8	13.1	6.5	0.3	−5.8	−6.2	−12.5	−18.8
9	10.8	−4.2	−10.8	−17.5	9.1	2.6	−4.0
Average	11.7	8.5*	2.1	−4.3	8.1*	1.6	−4.8
SD	1.3	5.5	5.6	5.8	6.9	6.6	6.4

Birds were fed either a dry or a wet diet. Values are given for presumed x values of 0, 0.25, and 0.50. $r_{CO_2\text{-IRGA}}$, rate of CO_2 production relative to infrared respiration gas analysis. *Average error of calculated rate of CO_2 production differs significantly from 0.

did not differ significantly between both diets, when calculated based on ^{2}H or ^{18}O dilution measured with IRMS ($P = 0.29$ and 0.48, respectively) and when based on ^{2}H and ^{18}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}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}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}H dilution spaces significantly exceeded those for ^{18}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}H dilution spaces exceeded those for ^{18}O by 1.1%, but this was not statistically significant ($P = 0.09$). In contrast, for the wet diet it was found that ^{2}H

dilution spaces significantly exceeded those for ^{18}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 $r_{H_2O_e}$ 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 $r_{H_2O_i}$ were 36.4 (SD = 9.3) and 37.0 (SD = 9.5) g/day, respectively. For the wet diet, corresponding values for $r_{H_2O_e}$ were 96.0 and 97.5 g/day, respectively (Table 1), and for $r_{H_2O_i}$ values were 100.6 (SD = 13.7) and 102.1 (SD = 13.8) g/day, respectively. Only for the dry diet, calculated $r_{H_2O_e}$ 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_{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 $r_{H_2O_i}$ values (based on IRMS and LS analyses), water economy index (WEI; g H_2O/l CO_2) values were calculated for each measurement by dividing $r_{H_2O_i}$ by the daily r_{CO_2}, as assessed from infrared respiration

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 H_2O/l CO_2, i.e., a 2.6-fold increase for the wet diet. To simplify the presentation of the results of the validation, $r_{CO_2\text{-IRMS}}$ and $r_{CO_2\text{-LS}}$ values are expressed as a relative deviation from the value obtained with the respiration gas analysis (Table 2).

For the dry diet, calculated r_{CO_2} 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 $r_{CO_2\text{-IRMS}}$ 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 $r_{CO_2\text{-IRMS}}$ and $r_{CO_2\text{-LS}}$, 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 $r_{CO_2\text{-IRMS}}$ and $r_{CO_2\text{-LS}}$, zero errors for the calculated mean r_{CO_2} were obtained at x levels of 0.43 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 r_{CO_2} to assumptions concerning x for the dry diet, the application of an x value of 0.58 (as derived from $r_{CO_2\text{-LS}}$) for the calculation of $r_{CO_2\text{-IRMS}}$ does not lead to an error significantly different from zero.

For the wet diet, calculated r_{CO_2} 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 $r_{CO_2\text{-IRMS}}$ and $r_{CO_2\text{-LS}}$ 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 r_{CO_2} by 4.3 and 4.8% for $r_{CO_2\text{-IRMS}}$ and $r_{CO_2\text{-LS}}$, 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 r_{CO_2} 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 r_{CO_2} are attributable to random analytic uncertainties, $r_{CO_2\text{-IRMS}}$ and $r_{CO_2\text{-LS}}$ 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, $r_{H_2O_e}$ 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 H_2O/l CO_2, 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), $x = 0.50$ 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 fecal 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 $r_{H_2O_e}$ 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, respectively, 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 $r_{H_2O_i}$ 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, $r_{H_2O_i}$ 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, $r_{H_2O_i}$ values were on average 43% below and 60% above prediction, respectively. Thus observed $r_{H_2O_i}$ values fell in the range as observed in captive and free-living birds.

Sensitivity of Calculated r_{CO_2} 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_{CO_2} 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 $r_{CO_2\text{-IRMS}}$ 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 $r_{CO_2\text{-LS}}$ 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_{CO_2} 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_{CO_2} 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_2O/l CO_2), assumptions concerning x exhibited rather little effect on the calculated r_{CO_2}, whereas, when they were fed the high diet

(WEI = 8.5 g H_2O/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 ^2H and ^{18}O turnover rates, and any small change in the assumed x will have a significant impact on the calculated r_{CO_2} 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_{CO_2} 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 ^2H 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_{CO_2} based on ^2H and ^{18}O turnover rates, as well as on ^2H and ^{17}O, a possibility that has been explored in the literature with radioactive (3) but not with stable isotopes.

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